α2-Chimaerin and Interacting Proteins in Axonal Guidance Signalling

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Abstract

Developing neurones require a dynamic cytoskeleton for newly forming processes to respond to guidance cues. The Rho GTPases, including Rac1, are very important in cytoskeleton remodelling. A GTPase activating protein (GAP) for Rac1, α2-chimaerin, participates in Semaphorin 3A (Sema 3A) and ephrin/EphA4 growth cone collapse pathways, and the SH2 domain is essential, suggesting phosphotyrosine interactions are involved. The activation of α2-chimaerin in collapse pathways may be through diacylglycerol binding to the cysteine rich C1 domain or via phosphotyrosine signalling. This investigation explores the interactions of α2-chimaerin and the role of tyrosine phosphorylation in retraction signalling pathways.

The Src family tyrosine kinase, Fyn, is involved in Sema 3A and ephrin A1/EphA4 signalling. Results showed that Fyn can bind to the GAP domain of α2-chimaerin and can phosphorylate α2-chimaerin activated by phorbol 12-myristate 13-acetate, Sema 3A or ephrin A1. Fyn was found to phosphorylate tyrosines 143 and 303 of α2-chimaerin. Over-expression of α2-chimaerin led to increased axon number in hippocampal neurones. Selected point mutations of α2-chimaerin were sufficient to affect axon branching in developing hippocampal neurones.

In COS-7 cells, the receptor EphA4 interacted with α2-chimaerin GAP domain when un-stimulated and with the SH2 domain when stimulated with the ligand ephrin A1. In N1E-115 cells permanently expressing EphA4, α2-chimaerin shRNA inhibited ephrin A1-induced cell rounding. The SH2 and C1 domains of α2-chimaerin were both necessary for ephrin A1/EphA4 cell rounding.

Another partner of α2-chimaerin is adaptor protein Nck1. The α2-chimaerin Nck1 binding region has been mapped and the binding may be sufficient to activate α2-chimaerin, allowing other partners to interact. A novel α2-chimaerin interacting protein Vps28, a component of the endosomal sorting complex required for transport-I, has been investigated in endocytosis using monoclonal antibodies and shRNA. These results together suggest an important regulatory role for α2-chimaerin and its partners in axonal guidance pathways.

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Abbreviations

Abl	Abelson
ADAM	A-Disintegrin-And-Metalloprotease
ADF	Actin-Depolymerisation Factor
ADP	Adenosine Diphosphate
Amp	Ampicillin
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BCR	Breakpoint Cluster Region
BSA	Bovine Serum Albumin
Cdc	Cell Division Cycle
Cdk	Cyclin Dependent Kinase
CHN1/2	Chimaerin gene 1/2
Comm	Commissureless
CRMP	Collapsin Response Mediator Protein
DAG	Diacylglycerol
DCC	Deleted in colorectal cancer
ddH_2O	Deionised purified water
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothretiol
E.coli	*Escherichia coli*
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ESCRT	Endosomal Sorting Complex Required for Transport
F-actin	Filamentous Actin
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum

FGF	Fibroblast Growth Factor
G-actin	Monomeric Globular Actin
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GIT	G-protein Coupled Receptor Kinase Interacting Targets
GPI	Glycosylphosphatidylinositol
GSK	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate
HCl	Hydrochloric Acid
IPTG	Isopropyl-Thio-D-Galactoside
JAK	Janus kinase
mDia	Diaphanous
MAP	Microtubule Associated Protein
MAPK	Mitogen Activated Protein Kinase
MRCK	Myotonic dystrophy kinase-Related Cdc42-binding Kinase
MTOC	Microtubule Organising Centre
MVB	Multivesicular Body
NF	Neurofilament
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartic acid
NPF	Nucleation Promotion Factors
NR2A	NMDA Receptor
NRTK	Non Receptor Tyrosine Kinase
N-WASP	Neural-Wiskott-Aldrich Syndrome Protein
PAGE	Polyacrylamide Gel Electrophoresis
PAK	p21 Activated Kinase
PB1	Phox and Bem1
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor

PH	Plexin Homology
PI3K	Phosphoinositide 3-Kinase
PIP	Phosphatidylinositol Phosphate
PKA	cAMP Dependent Protein Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethyl-Sulfonyl Fluoride
PP1/2A	Protein Phosphatase 1/2A
PSI	Plexins, Semaphorins and Integrins domain
PTB	Phosphotyrosine Binding Domain
PTP	Protein Tyrosine Phosphatase
PTK	Protein Tyrosine Kinase
PVDF	Polyvinylidene Fluoride
Rac	Ras-related C3 Botulinum Toxin Substrate
Rho	Ras Homologous Member A
Robo	Roundabout
ROCK	Rho-Kinase
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SFK	Src Family Kinase
SH2	Src Homology 2 Domain
SH3	Src Homology 3 Domain
TEMED	N, N, N', N'-Tetramethylethylenediamine
WASp	Wiskott-Aldrich Syndrome protein
WAVE	WASP family verprolin homologous protein
Vps	Vacuolar Protein Sorting Protein

Chapter 1.0:

Introduction

1.0 Introduction

In brain development, neuronal cells originate from proliferative layers of progenitor cells from which they migrate to their final positions and differentiate, acquiring distinct neuronal identities and connections (Donahoo and Richards, 2009; Kwan *et. al.*, 2012). As neurogenesis progresses, neurones are generated sequentially and migration occurs in an inside-first, outside-last manner. Mechanisms regulating neuronal migration include cell-cell interactions and cytsokeletal dynamics. The actin and microtubule cytoskeleton, the cellular scaffolding, forms dynamic structures allowing individual neurone subtypes to differentiate and develop complex morphology. Neurones form a highly ordered network of connectivity in the adult central and peripheral nervous system, supported by non-neuronal glial cells, including oligodendrocytes, astrocytes and Schwann cells. Developing axons and dendrites respond to specific guidance signals. Attractive and repulsive signalling molecules are displayed by neighbouring cells, secreted soluble factors or in the extracellular matrix. Their various membrane receptors activate intracellular signalling pathways, transmitting the external guidance stimulus to proteins that reorganise the cytoskeleton. Intracellular signalling pathways involve multiple proteins including the Rho GTPase family of proteins, which act as molecular switches. Rac1 is a member and like the rest of the RhoGTPase family its interactions and functions are tightly regulated by GTPase activating proteins (GAPs) and Guanine-nucleotide exchange factors (GEFs). The Rho GTPases act via a range of effector proteins playing a pivotal role in coordinating dynamic changes in the actin cytoskeleton.

This study investigates α2-chimaerin, a neuronal GAP specific for Rac1 which enhances intrinsic Rac GTPase activity, thereby down-regulating Rac1. GAPs are themselves tightly regulated multi-domain signalling proteins. This study aims to investigate α2-chimaerin regulation and interactions with partner proteins that contribute to the complex orchestration of repulsive guidance pathways in developing neurones.

1.1 The Cytoskeleton

The cytoskeleton comprises three major components; actin filaments, microtubules and intermediate filaments and is crucial in many dynamic cell processes. These include cell motility, cell polarity, cell division, intracellular trafficking and endocytosis (Pollard and Cooper, 2009).

1.1.1 Actin Filaments

Actin assembly provides a force for re-shaping cells and cell movement. Monomeric globular (G-actin) polymerises to form filamentous actin (F-actin). Actin filaments are polar structures with a slow growing pointed end and a fast growing barbed end and are mainly cross-linked into aggregates and bundles. During polymerisation ATP-actin joins the barbed end of a filament, the ATP molecule is hydrolysed causing a conformational change and trapping ADP inside the actin monomer (Pollard *et. al*., 2000). Although ATP bound to actin stabilises the molecule, it is not required for polymerisation as such, but seems to indicate filament age and initiate processes for depolymerisation (Pollard and Borisy, 2003).

Treadmilling, first described by Wegner in 1976, occurs as ATP-bound actin joins filaments at the barbed end and ADP-bound actin dissociates from the pointed end at the

same rate, due to ATP-actin and ADP-actin having the same kinetic constant (Frederick *et. al.*, 2008). Treadmilling is a slow process that has been visualised by fluorescence microscopy (Fujiwara *et. al.*, 2002) and therefore other factors, such as actin binding proteins, are required to regulate polymerisation and accelerate the process in migrating cells (Pollard and Borisy, 2003).

At the leading edge of migrating cells, the barbed ends of actin filaments drive lamellipodia (sheet-like) and filopodia (rod-like) extensions. Actin filaments found in lamellipodia are short and highly branched. In contrast, filopodia consist of longer, un-branched, unipolar, parallel bundles of actin filaments (Le Clainche and Carlier, 2008). Protrusion of the leading edge is driven by the lamellipodium and the lamella (Svitkina and Borisy, 1999). Fluorescence speckle microscopy studies have shown these two distinct but overlapping actin systems at work, with the lamellipodium as fast but short-lived speckles and the lamella as slow-moving, longer-lived speckles (Ponti *et. al.*, 2004; Iwasa and Mullins, 2007). The formation of lamellipodia and filopodia in the same area of the cell may be explained by two different nucleation mechanisms involving the Arp2/3 complex and formins, creating two actin networks (Le Clainche and Carlier, 2008).

The Arp2/3 complex comprises seven proteins, actin-related Arp2 and Arp3 and ARPC1-5 (Machesky *et. al.*, 1994). In its purified form Arp2/3 cross-links the pointed end of one actin filament to the side of another filament at a 70 degree Y-branch angle (Mullins *et. al.*, 1998). Like actin, Arp2 and Arp3 bind to ATP, causing conformational changes to the Arp2/3 complex (Goley *et. al.*, 2004; Zencheck *et. al.*, 2009).

The Arp2/3 complex is a weak actin nucleator, but it can be activated by nucleotide binding and interactions with nucleation promoting factors (NPFs), such as the WASp (Wiskott-Aldrich Syndrome protein)/Scar family, of which there are five members, WASp, N-WASP (Neural-Wiskott-Aldrich Syndrome Protein) and Scar/WAVE (WASP family verprolin homologous protein) proteins 1-3 (Higgs, 2001). The majority of mammalian NPFs, including the WASp/Scar family, activate the Arp2/3 complex through a VCA domain. This consists of a WASP-homology-2 or verprolin homology domain which binds G-actin, a central region, and an acidic region which interacts with the Arp2/3 complex (Campellone and Welch, 2010). Other domains of N-WASP interact directly with Cdc42 (via the CRIB domain/GTPase binding domain (GBD)) (Aspenstrom *et. al.,* 1996), Phosphatidylinositol Phosphate$_2$ (PIP_2) (to the basic region) (Miki *et. al.,* 1996) and Src Homology (SH)3 domain proteins including Nck (via the proline rich region) (Rivero-Lezcano *et. al.,* 1995). N-WASP is auto-inhibited through association of GBD with VCA unless activated by multiple inputs, including Cdc42 (Padrick and Rosen, 2010). WAVE acts downstream of Rac1, stimulating the Arp2/3 complex (Miki *et. al.,* 1998). WAVE proteins are constitutively part of a complex with PIR121 (also known as Sra1 or CYFIP2), Nck associated protein 1, Abelson (Abl) interacting protein and HSPC300 (Innocenti *et. al.,* 2004), in which inhibition of WAVE by PIR121 is relieved by active Rac, and WAVE is also phosphorylated (Chen *et. al.*, 2010).

In contrast to the Arp2/3 complex, other actin nucleators produce linear filaments, the most studied of these being the formins. The formins contain conserved formin-homology domains FH1 and FH2 and alternatively from the Arp2/3 complex, catalyse polymerisation of filaments at the barbed end. It has been suggested that formins promote

polymerisation by stabilising spontaneously formed actin dimers/trimers (Pring *et. al.*, 2003). More recent additions to identified nucleators involved in actin monomer recruitment to filaments include Spire, Cordon bleu and Leiomodin (Chesarone and Goode, 2009).

Rho-family GTPases are extremely important in actin dynamics, acting through their many effector proteins which include NPFs and kinases, and they play a key role in NPF regulation (Section 1.3). Early studies showed Rho is responsible for the formation of stress fibres and focal adhesions, Rac for lamellipodia and ruffles (Ridley *et. al.*, 1992; Ridley and Hall, 1992), and Cdc42 for formation of filopodia (Nobes and Hall, 1995; Kozma *et. al.*, 1995). The cellular activities of the RhoGTPases are spatially and temporally co-ordinated. Recently, a live-cell study of RhoA, Rac and Cdc42 activation suggests Rho acts transiently to initiate protrusive events with sequential activation of Cdc42 and Rac (Machacek *et. al.*, 2009).

1.1.2 Actin-Binding Proteins

In addition to the Arp2/3 complex, formins and the newer actin nucleators, there are hundreds of other actin-binding proteins, with functions involved in forming, stabilising and depolymerising actin structures (Dominguez, 2009). Amongst these are Actin-depolymerisation factor (ADF) and cofilin, which are involved in severing actin filaments and the rapid turnover of actin filament barbed ends that drive forward the leading edge of cells (Carlier *et. al.*, 1999). Profilin is an actin monomer binding protein which enhances the exchange of ADP to ATP and the rate of actin treadmilling (Yarmola and Bubb, 2006). Gelsolin and Capping Protein work together to regulate the barbed end of

actin filaments (Barkalow *et. al.,* 1996). Cross-linking proteins, such as tropomyosin, strengthen actin filaments by binding along the length of a filament. Fimbrin and α-actinin bundle filaments and spectrin attaches the sides of filaments to the plasma membrane (Uribe and Jay, 2009). Myosins I, V and VI are motor proteins implicated in movement of vesicles in the actin cortex (Eichler *et. al.,* 2006; Buss and Kendrick-Jones, 2008; Kim and Flavell, 2008). Focal contacts, points of cell attachment and signalling complexes, are controlled by the integrin family of proteins, including talin and vinculin, which also associates with α-actinin. Tyrosine kinases Src and focal adhesion kinase (FAK) are involved in focal contact formation (Section 1.3.4).

1.1.3 Microtubules

Microtubules are made up of GTP-binding α- and β-tubulin heterodimers that form polarised helical structures, which can be stabilised by the pointed end joining a microtubule organising centre (MTOC) (Desai and Mitchison, 1997). Microtubules play a major role in cell polarisation. In hippocampal neurones, microtubules in axons are arranged in a uniform polarity with the barbed end directed towards the growth cone, whereas microtubules in dendrites contain a mixed orientation of polarity (Baas *et. al*., 1988). Axons also contain more stable acetylated microtubules, whilst dendritic microtubules are less stable and more dynamic (Witte and Bradke, 2008). In developing neurones, microtubules stabilize in one neurite which then goes on to become the axon (Witte *et. al*., 2008).

Microtubule-associated proteins (MAPs) are important in microtubule dynamics and in mediating microtubule interactions. There are two major classes of MAPs; high

molecular weight proteins of 100-200 kDa, MAP-1 and MAP-2, and Tau proteins, which have molecular weights of around 60 kDa. Both classes of protein contain a microtubule binding domain and a domain which links microtubules to other cell components. Neuronal MAPs bind to microtubules and crosslink adjacent tubulin subunits to stabilise the structures and promote the polymerisation phase (Desai and Mitchison, 1997). Tau is mainly located in axons with the more stable microtubules and MAP-2 is associated with dendritric microtubules (Mandelkow and Mandelkow, 1995). A further class of microtubule associated protein are collapsin response mediator proteins (CRMP1-5). These dihydropyrimidinase related proteins promote axon outgrowth and play a role in microtubule stability (Inagaki *et. al*, 2001). CRMP-2 C-terminal is phosphorylated by Cyclin dependent kinase (Cdk) 5 and Glycogen Synthase Kinase (GSK)-3β (Brown *et. al.*, 2004; Uchida *et. al.*, 2005). The interaction of CRMP-2 with microtubules is dynamically regulated through phosphorylation (Lin *et. al.*, 2011) similarly to Tau (Mandelkow and Mandelkow, 2012). CRMP-2 is an α2-chimaerin binding partner (Brown *et. al.*, 2004).

1.1.4 Intermediate Filaments

In contrast to globular actin and tubulin monomers, intermediate filament (IF) proteins are apolar and highly elongated polymers of fibrous proteins. IFs provide mechanical strength and stability to the cell by cross linking to each other, to microtubules and to actin via plectin or spectrins, helping to protect cells against mechanical stresses (Svitkina *et. al.*, 1996). IFs differ between cell types, for example, the main IF in astrocytes is glial fibrillary acidic protein (Liem and Messing, 2009). Neuronal cell IFs

consist of three proteins, NF (neurofilament)-L, which provides the backbone of the neurofilament for NF-M and NF-H (Fuchs and Cleveland, 1998), and α-internexin (Fliegner *et. al.,* 1990; Kaplan *et. al.,* 1990). Septins are a family of filament forming GTP-binding proteins, which bind to membranes, actin and microtubules to stabilise cells and participate in cellular processes involving the cytoskeleton (Silverman-Gavrila and Silverman-Gavrila, 2008).

1.2 Protein Phosphorylation and Signal Transduction

Post-translational protein modifications provide transient regulation of protein-protein interactions in signal transduction. Phosphorylation is the commonest modification, causing conformational changes, activation or deactivation and regulating or promoting binding sites (Ubersax and Ferrell, 2007). There are several hundred kinases which phosphorylate tyrosine or serine and threonine residues (Chico *et. al.,* 2009).

1.2.1 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are an important class of receptor for guidance molecules and extracellular ligands involved in the development of the nervous system. There are several hundred RTKs encoded in the human genome. The Trk family are receptors for nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophins (NTs). NGF is required for the survival and differentiation of sympathetic neurones (Levi-Montalcini, 1987). BDNF is important for support of dendritic structure and neuronal plasticity (Martinowich *et. al.*, 2003; Jiang *et. al.*, 2008). NTs are growth factors which are vital in neuronal network formation and plasticity (Huang and

Reichardt, 2003). The Eph family of receptors, which are receptors for ephrins, are discussed in Section 1.4.2. RTKs and their respective ligands are crucial in the development and regulation of neurones.

RTKs such as Trks, are membrane bound proteins with typically three domains; an extracellular glycosylated ligand binding receptor domain, a transmembrane domain comprising a single transmembrane α-helix and an intracellular domain that includes an intrinsic tyrosine kinase domain (Hubbard, 2002). Following ligand binding, RTKs dimerise and autophosphorylate, initiating intracellular signalling cascades. Autophosphorylated tyrosines act as high affinity binding sites for intracellular signalling proteins, which usually contain single or combinations of phosphotyrosine binding (PTB), Src homology (SH) 2 and SH3 domains (Pawson, 2002). Recruitment and tyrosine phosphorylation may activate intracellular signalling proteins, for example phospholipase Cγ (PLCγ), which catalyses hydrolysis of phosphatidylinositol (4, 5) bisphosphate with formation of inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG) (Segal, 2003). RTK activity is limited by protein tyrosine phosphatases (Östman and Böhmer, 2001) and endocytic trafficking (Wiley and Burke, 2001).

1.2.2 Non-Receptor Tyrosine Kinases

Non-receptor tyrosine kinases include the Src, Abl and Janus families of kinases. In the nervous system, Abl kinases are involved in regulating neuronal morphogenesis, controlling cytoskeletal dynamics in neurones and regulating synaptic structure and function (Moresco and Koleske, 2003). The Janus family are involved in JAK-STAT (Janus kinase - signal transducer and activator of transcription proteins) signalling,

downstream of cytokines in the immune system, and transmit stress signals from the plasma membrane to the nucleus (Boengler *et. al.*, 2008). The Janus family of non-receptor tyrosine kinases consists of four members- JAK1, 2 and 3 and Tyk2. Their domain structure consists of a FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain, thought to be important in localisation of the protein, an SH2-like domain, which lacks the phosphotyrosine binding of conventional SH2 domains, a kinase-like domain and a protein tyrosine kinase domain (Wilks, 2008).

The most studied non-receptor tyrosine kinases are the Src family kinases (SFKs) which play a fundamental role in cell adhesion, morphology and motility (Roskoski, 2004), as well as in proliferation and differentiation in the development of neurones (Kalia *et. al.*, 2004). There are eight members, comprising Src, Fyn (Section 1.2.3), Yes, Lck, Lyn, Blk, Fgr, Hck, and three SFK related kinases Srm, Frk and Brk. SFKs function in diverse signalling pathways including the semaphorin (sema) 3A and ephrin/Eph collapse pathways (Sasaski *et. al.,* 2002; Liu *et. al.*, 2004; Shi *et. al.*, 2007). SFKs are also associated with various downstream targets including PI3-kinase, PLC, FAK and p190 rhoGAP (Frame, 2004; Ingley, 2008).

1.2.3 Fyn in Axonal Guidance

Fyn tyrosine kinase, a member of the Src family, comprises an N-terminal (SH4) domain containing acylation sites, a unique region, SH3 and SH2 domains, an SH1/kinase domain and a C-terminal regulatory tail (Martin, 2001). The most N-terminal glycine can be myristoylated, occurring co-translationally on free polysomes. This acylation anchors Fyn to the plasma membrane (Resh, 1998). Palmitoylation can occur on the most N-

terminal cysteine, which may target Fyn to lipid raft microdomains and directs the remaining SH4 region away from the membrane, possibly for Fyn interaction with target proteins (Rawat and Nagaraj, 2010). Fyn is held in an inactive closed conformation by intramolecular binding of SH2 to a phosphorylated tyrosine (Y531) in the C-terminal regulatory domain and SH3 with the linker region between SH2 and SH1. Dephosphorylation of the regulatory tyrosine or binding of SH2 or SH3 domains by external ligands alters Fyn conformation to an open 'active' state. Additionally, intermolecular autophosphorylation of tyrosine 420 in the SH1/kinase domain stabilises the active state (Roskoski, 2004). It is well recognised that Fyn is important in axon-glial signal transduction and processes needed for oligodendrocyte maturation and CNS myelination (Krämer-Albers and White, 2011). Fyn tyrosine kinase has been shown to be involved in the sema 3A, ephrin-Eph and netrin-1 neuronal signalling pathways (Sasaki *et. al.*, 2002; Liu *et. al.,* 2004; Shi *et. al.,* 2007; Zhou *et. al.*, 2008) and is essential for normal brain development (Goto *et. al.,* 2008). In the sema 3A pathway Fyn phosphorylates Cdk5 Y15, activating Cdk5, mediating growth cone collapse and axonal guidance (Sasaski *et. al.,* 2002), and plays a role in dendritic spine maturation (Morita *et. al.,* 2006). Fyn has been shown to phosphorylate CRMP-2 (Uchida *et. al.,* 2009), a component of the sema 3A pathway (Goshima *et. al.,* 1995), required for sema 3A induced growth cone retraction (Brown et al 2004**;** Uchida *et. al.,* 2009). Figure 1.1 shows a representation of Fyn in sema 3A signalling. Several GAPs have been reported to be regulated by Fyn phosphorylation. Fyn phosphorylates p190 RhoGAP in the regulation of oligodendrocyte differentiation (Wolf *et. al.,* 2001), Fyn phosphorylation of p250RhoGAP enhances its GAP activity (Taniguchi *et. al.,* 2003), and in contrast,

TCGAP activity is suppressed by Fyn phosphorylation of its GAP domain (Liu *et. al.*, 2006).

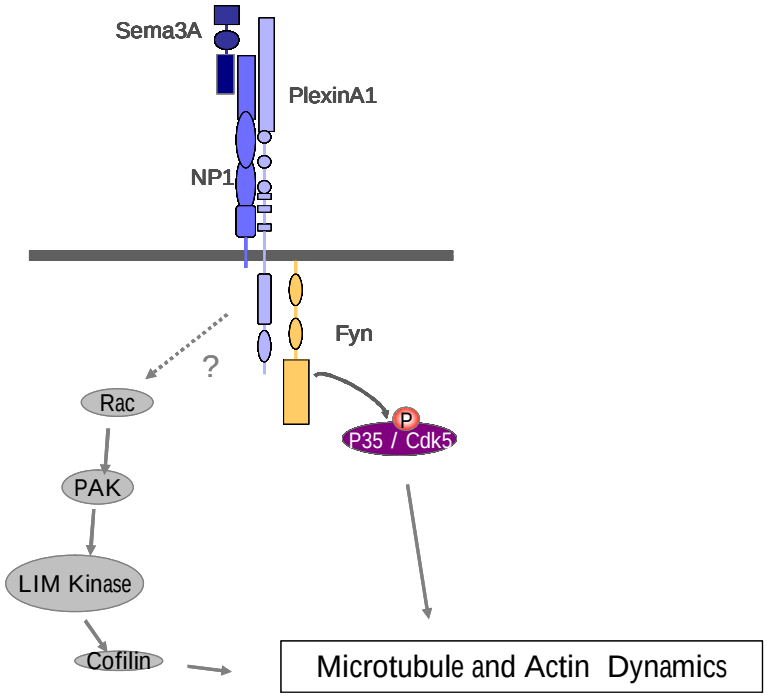

Figure 1.1: Fyn in sema 3A signalling A representation of Fyn in the sema 3A pathway. The ligand sema 3A binds to the receptor complex of neuropilin 1 (NP1) and Plexin A1, which transmits the signal across the plasma membrane. Fyn binds plexin, which phophorylates Cdk5 at Y15, downstream signalling through Rac leads to cytoskeletal remodelling. (Adapted from Sasaki *et. al.*, 2002.)

1.2.4 Cdk5 in Neurones

Cdk5 is one of a large family of proline-directed serine/threonine kinases and has a fundamental role in neurones; during development in neuronal positioning and migration in establishing cortical layers, in synapse formation and axonal patterning, as well as regulating membrane dynamics and intracellular trafficking (Smith and Tsai, 2002). Cdks are generally reliant on a cyclin for their activation; Cdk5 is an exception and is activated

by two partners, p35 and p39. Although not activated by cyclins, Cdk5 does have the ability to bind to cyclin D (Xiong *et. al.*, 1997).

Calpain is known to cleave Cdk5 activator p35 to a constitutively active form, p25, causing hyperactivity of Cdk5. This can result in hyperphosphorylation of Cdk5 substrates such as tau, MAPs and neurofilaments, which leads to neuronal cell death and has been implicated in Alzheimer's disease (Kesavapany *et. al.*, 2004). Cdk5 is also thought to play a role in the early stages of memory formation and in learning (Angelo *et. al.*, 2006). Cdk5 acts downstream of EphA4 in ephexin dependent retraction of dendritic spines (Fu *et. al.*, 2007), and plays a role in sema 3A signalling (Sasaki *et. al.*, 2002). Cdk5 and p35 interact with the C-terminal of α2-chimaerin (Qi *et. al.*, 2004; Brown *et. al.*, 2004).

1.2.5 Serine/Threonine Kinases, PKA, PKC and CamK11

Three other well characterised serine/threonine kinases enriched in the brain and involved in receptor and ion channel modulation, neurotransmitter synthesis and release, and synaptic strength and plasticity (among other functions) are Cyclic AMP-dependent protein kinase A (PKA); calcium/phospholipid-dependent protein kinase C (PKC) (Section 1.2.6); and calcium/calmodulin-dependent kinase II (CaMKII). Each of these kinases becomes active when their respective secondary messengers displace an autoinhibitory domain, freeing the catalytic subunit/domain to phosphorylate specific protein substrates (Domańska-Janik, 1996).

PKA is a tetramer formed of two regulatory subunits containing two cAMP binding sites and two catalytic subunits. When cAMP binds to the regulatory subunits, the

homodimeric regulatory core is released from the catalytic subunits thus activating them (Dai *et. al*., 2009). The cAMP/PKA pathway plays a major role in regulating the corticotrophin releasing factor gene in hypothalamic neurones, which controls the stress response (Suda *et. al.*, 1985; Seasholtz *et. al.*, 1988; Spengler *et. al*., 1992).

CaMKII kinases are large multimeric enzymes, derived from four genes, producing six-twelve kinase subunits each. The α and β genes are neuronal isoforms, whereas the γ and δ genes are more broadly expressed. Calmodulin binds to the regulatory domain of CaMKII and activates the kinase by allowing ATP and protein substrates to bind to the catalytic region. Calmodulin binding is further assisted by the autophosphorylation of a threonine residue in the autoinhibitory domain. After calmodulin dissociation, CaMKII is still active, subsequent autophosphorylation of different residues is required for kinase deactivation (Domańska-Janik, 1996). CamKII is enriched in postsynaptic densities of excitatory synapses and is associated with long-term potentiation (Fukunaga *et. al*., 1993; Lledo *et. al*., 1995; Barria *et. al*., 1997).

1.2.6 PKC Family and C1 Domain in Regulation

The PKC super-family of serine/threonine kinases play a vital role in signal transduction pathways and are regulated by lipids in a manner similar to chimaerins. PKCs phosphorylate a wide range of substrates implicated in neurotransmitter release, cell proliferation, synaptic remodelling and gene expression (Ron and Kazanietz, 1999; Amadio *et. al.*, 2006). PKCs have four conserved domains, C1-4, and five variable regions V1-5. C1 is the cysteine-rich, DAG binding domain additionally found in chimaerins and several other proteins, they also specifically bind phosphatidylserine

(Kheifets and Mochly-Rosen, 2007; Johnson *et. al.*, 2000). C1 domains are important in PKC regulation, they are involved in recruiting proteins to membranes and have a conserved motif $HX_{12}CX_{13\text{-}14}CX_2CX_4HX_2CX_7C$, where X is any amino acid (Hommel *et. al.*, 1994). C2 domains are most well known as calcium sensors and phosphatidylserine-binding domains (Cho and Stahelin, 2006). The ATP-binding C3 domain and the substrate-binding C4 domain form the catalytic region (Amadio *et. al.*, 2006).

PKCs are split into three subfamilies based on the N-terminal regulatory domain structures, which dictate the different cofactors of PKC isoenzymes (Newton, 2001). There are ten PKC family members with four conventional PKCs, containing a C1 and a C2 domain, which require DAG, calcium and phosphatidylserine as their cofactors. Four novel PKCs also have C1 and C2 domains, although the C2 domain lacks calcium binding, they require DAG and phosphatidylserine as cofactors. There are two atypical PKCs that have an atypical C1 domain, lacking DAG binding and do not have a C2 domain, they only require phosphatidylserine as a cofactor (Newton, 2003). Atypical PKCs contain an additional protein-protein interaction domain, Phox and Bem1 (PB1), which interacts with other PB1 domain containing scaffolding proteins (Steinberg, 2008). N-terminal to PKC C1 domain sits an autoinhibitory/pseudosubstrate domain, which maintains PKC in an inactive conformation. The sequence of the autoinhibitory domain resembles a PKC substrate; but contains an alanine instead of a serine/threonine phosphoacceptor site (Steinberg, 2008). Activation is also related to translocation of PKCs to different intracellular sites and subsequent serine/threonine phosphorylation of downstream substrates (Amadio *et. al.*, 2006).

For conventional PKCs, signals that increase intracellular calcium and DAG result in calcium binding the C2 domain, anchoring PKC to the plasma membrane (Nalefski and Newton, 2001; Schaefer *et. al.*, 2001). However, this does not activate PKC, activation occurs when DAG binds the C1 domain, the pseudosubstrate is released and downstream binding and signalling takes place (Nishizuka, 1995; Toker, 1998; Newton, 2001). Hydrolysis of phosphatidylinositol-(4,5)-bisphosphate by PLC produces DAG and inositol-1, 4, 5-trisphosphate (IP_3). IP_3 stimulates the release of calcium from intracellular stores. Conventional PKCs are recruited to the membrane and the calcium ions bridge the C2 domain to phosphatidylserine. PKC is activated at the membrane by DAG binding the C1 domain. The exposed surface of phorbol ester (DAG)-bound C1 domains is hydrophobic and buried in the lipid bilayer of the membrane (Corbalán-García and Gómez-Fernández, 2006). The hydrophobic pocket that binds DAG or phorbol esters is formed by a zinc finger coordinating with cysteine and histidine residues and their hydrophobic side chains (Hurley *et. al.*, 1997).

The crystal structure of PKCβ11 revealed a two-step activation of its autoinhibited conformation. The active site is accessible to the substrate, but the ATP-binding side chain of the conserved NFD motif is displaced, preventing full activation. The C1B domain holds the NFD helix in a less active conformation, which is reversed by membrane binding (Leonard *et. al.*, 2011).

Several other proteins contain a C1 domain, demonstrating an important role for DAG in diverse signalling pathways. In addition to PKCs and chimaerins, other C1 domain containing proteins include PKD, DAG kinases, the exchange factors RasGRPs, munc 13 and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (Brose and

Rosenmund, 2002; Tan *et. al.,* 2001). The mechanism of DAG binding to the C1 domain will differ between proteins and some C1 domains (such as in atypical PKCs and Raf) are non-functional in phorbol ester binding (Colón-González and Kazanietz, 2006; Hall *et. al.*, 2005).

1.2.7 Protein Phosphatases

Protein phosphatatases are important in reversing the action of a kinase by removing the phosphate group. As with kinases, there are receptor and non-receptor tyrosine phosphatases and phosphatases that dephosphorylate tyrosines or serines and threonines. Protein tyrosine phosphatases have been linked to cell adhesion receptors, growth factor receptors and also to ion/neurotransmitter-gated channels (Arregui *et. al.,* 2000). Receptor protein tyrosine phosphatases have significant roles in axonal guidance, involving target recognition, as inhibitory guidance cues and in synapse formation (Ensslen-Craig and Brady-Kalnay, 2004). Protein phosphatase 1 (PP1) and PP2A are families of the most abundant serine/threonine phosphatases (Virshup and Shenolikar, 2009). Defective PP2A expression has been associated with the phospho-Tau pathology seen in Alzheimer's disease (Sontag *et. al.,* 2004).

1.3 GTPase Superfamily - Rho GTPases

Monomeric G proteins or small GTP-binding proteins with molecular weights between 20 and 40 kDa make up a superfamily with more than one hundred members classified into at least five families; Ras, Rho, Rab, Arf and Ran. The GTPases act like molecular switches, when bound to GTP they are 'on' and in an active state and engage with effector proteins, and when bound to GDP they are 'off' (inactive). Regulation of GTPases switching between the active and inactive states is tightly controlled by other proteins, which also contribute to specificity as described below. This study involves the Rho family of GTPases, which are involved in cytoskeletal reorganisation and gene expression (Takai *et. al.*, 2001). The most studied members of the Rho GTPase family are RhoA, Rac1 and Cdc42. Rho GTPases are vital in controlling signalling pathways affecting the actin cytoskeleton (Schmandke *et. al.*, 2007), and crucial in neuronal migration and axonal guidance (Kawachi and Hoshino, 2008). RhoGTPases contain a C-terminal CAAX box which is modified posttranslationally by geranylgeranylation increasing hydrophobicity and membrane association (Sebti and Der, 2003).

1.3.1 Rho GTPase Regulation

1.3.1.1 Guanine-nucleotide Exchange Factors

GEFs act by displacing a GDP molecule from the GTPase enabling exchange for a GTP molecule, thereby activating the GTPase. GEFs are themselves tightly regulated by specific upstream signals, determining the activation of a GTPase (Rittinger, 2009). Some GEFs are specific for one GTPase, whereas others have broader substrate specificity (Takai *et. al.*, 2001). Identified Rho GEFs contain a Dbl-homology (DH) domain and a

pleckstrin homology (PH) domain in tandem, or alternatively, an unrelated domain identified in DOCK (dedicator of cytokinesis) proteins, which activates Rac (or Cdc42) (Bos *et. al.,* 2007).

1.3.1.2 Guanine-nucleotide Dissociation Inhibitors (GDIs)

GDIs have wide substrate specificity and maintain GTPases in their inactive state by inhibiting the exchange of GDP for GTP by preventing GDP dissociation, and influence cellular location by maintaining the GTPase in the cytosol (Sasaki and Takai, 1998). RhoGDI binding masks the C-terminal membrane targeting CAAX moiety (Michaelson *et. al.,* 2001).

1.3.1.3 GTPase Activating Proteins

GAPs stimulate intrinsic GTPase activity by hydrolysing GTP to GDP and inorganic phosphate and switching the GTPase to the inactive state. There are more than 70 Rho GAPs in eukaryotes (Bernards, 2003), outnumbering the Rho GTPases which they regulate, as with GEFs. GAPs have multiple domains and may provide specificity via selective tissue expression, GTPase specificity, regulation of different signalling pathways and/or acting as recognition modules (Tcherkezian and Lamarche-Vane, 2007). GAPs, like the GEFs can have a single substrate or a range of substrates. Rho GAPs are involved in a wide variety of cellular functions, for example, MgcRacGAP is involved in cytokinesis by varying its location throughout cell cycle progression (Hirose *et. al*., 2001; Kitamura *et. al.*, 2001) and p190 RhoGAP is important in neuronal differentiation, where it facilitates axon stability and branching (Billuart *et. al.,* 2001). Overexpression promotes neuritogenesis and p190RhoGAP, regulated by Src, acts downstream of cell

adhesion molecules (Brouns *et. al.*, 2001). P190RhoGAPs, comprising p190B RhoGAP (ARHGAP5) and p190A RhoGAP (ARHGAP35), are important in antagonizing RhoA activity in various cell functions including cytokinesis (Mikawa *et. al.*, 2008). Inhibition of RhoA via Arg and p190RhoGAP promotes dendritic spine maturation (Sfakianos *et. al.*, 2007). In the hippocampus, the Rac GAP family consisting of the breakpoint cluster region gene product (BCR) and Abr (active Bcr-related) have been implicated in excitatory synapse regulation, where mice lacking BCR or Abr showed a decrease in the maintenance (but not induction) of long term potentiation (Oh *et. al.*, 2010). Chimaerins Rac GAPs are discussed later (Section 1.5).

GEFs and GAPs are multi-domain proteins, which in addition to the conserved catalytic Rho GAP or GEF domain, contain various other domains. These include SH3, SH2, BAR, C1 and PH domains which combine additional functions to GEF and GAP proteins such as localisation targeting and scaffolding (Tolias *et. al.*, 2011). The regulation of GEFs and GAPs is tightly controlled and includes protein-protein or protein-lipid interactions, second messenger binding, post-translational modifications and autoregulation (Jacobs and Hall, 2005). Effects of regulation may consist of translocation to the location of the GTPase, release of an auto inhibited state and/or allosteric changes in the catalytic domain (Bos *et. al.*, 2007). Figure 1.2 shows Rho GTPase cycle regulation.

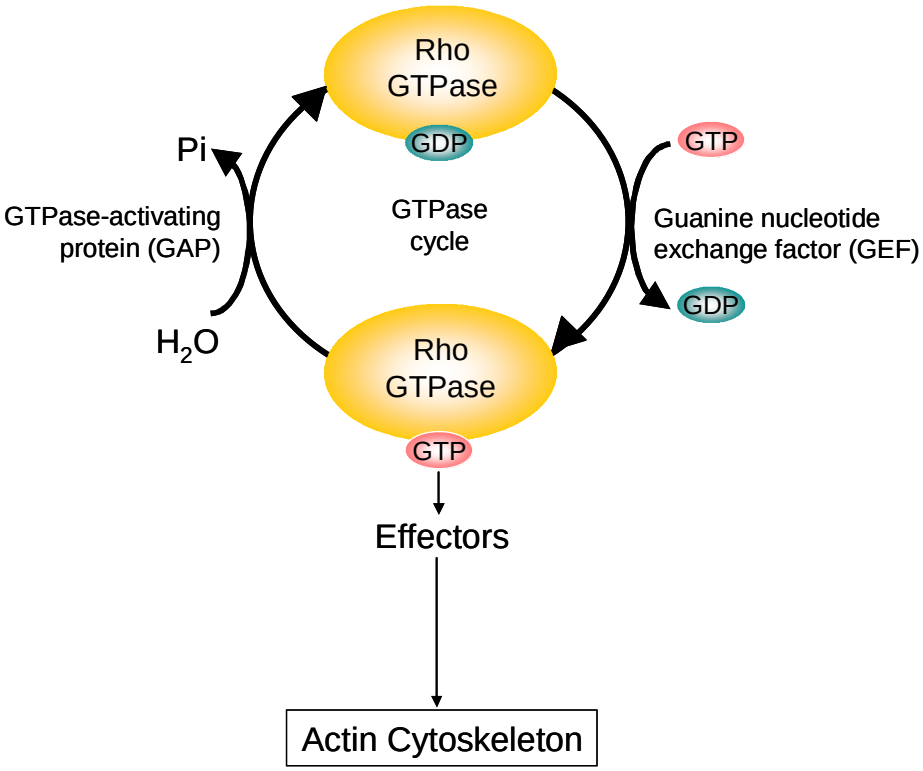

Figure 1.2: Rho GTPase regulation Rho GTPases act like molecular switches, being inactive when bound to GDP and activated when bound to GTP, associating with their effectors to induce changes on the actin cytoskeleton. GAPs inactivate Rho GTPases by hydrolysing GTP molecules to GDP and GEFs activate Rho GTPases by exchanging GDP molecules for GTP.

1.3.2 Rho GTPase Regulation of the Actin Cytoskeleton

Downstream of extracellular signals RhoA, Rac1 and Cdc42 coordinate reorganisation of the cytoskeleton, which is important in many cell processes including; cell growth, membrane trafficking, development, axon guidance, axon extension, cell shape changes, motility, adhesion and cytokinesis. The Rho GTPases are involved in these processes either directly or indirectly by regulation of the cytoskeleton and altering gene expression (Takai *et. al.*, 2001). Figure 1.3 is a summary diagram of the main RhoGTPases, their effector proteins and how they affect cell processes.

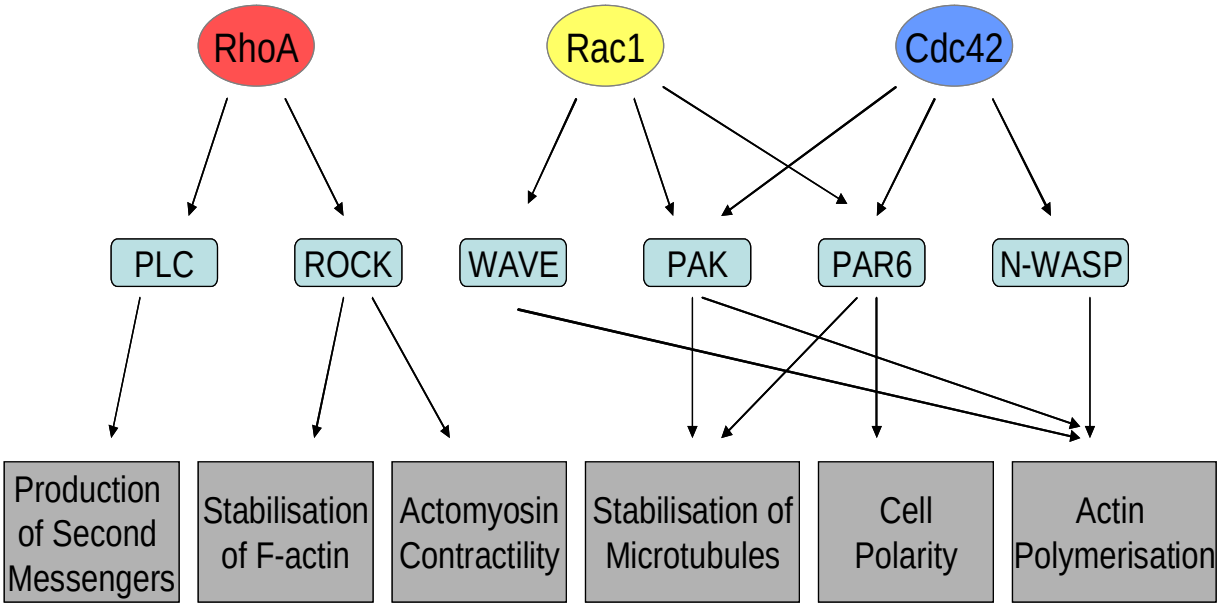

Figure 1.3: Rho GTPases in cell processes (adapted from Iden and Collard, 2008) A summary diagram showing Rho GTPase involvement in multiple cell processes, particularly in the organisation of the cytoskeleton. The most well studied Rho GTPases are RhoA, Rac1 and Cdc42, some of their main effector proteins (light blue) are shown in the diagram.

1.3.3 Morphological Effects of Rho Proteins

The role of RhoA in the reorganisation of the actin cytoskeleton was first shown in Swiss 3T3 fibroblastic cells. RhoA is essential for the formation of stress fibers and focal adhesions induced by LPA (Ridley and Hall, 1992). RhoA promotes focal adhesions and stress fibres leading to a contractile cell phenotype acting through its effectors Rho-kinase (ROCK) and diaphanous (mDia), which are essential in this process. ROCK phosphorylation of the regulatory serine 19 of MLC2 (Vicente-Manzanares *et. al.*, 2009) and of myosin phosphatase (i.e. the myosin-binding subunit MBS or MYPT1 of myosin phosphatase) promotes increased contractility (Leung *et. al.*, 1996; Matsui *et. al.*, 1996;

Nakano *et. al.,* 1999; Watanabe *et. al.,* 1999). ROCK also phosphorylates and activates LIM kinase, which subsequently phosphorylates cofilin, resulting in actin filament stabilisation (Maekawa *et. al.,* 1999). RhoA and ROCK activity are required in migrating cells for the regulation of tail retraction (Pertz *et. al*., 2006).

Rac1 stimulates lamellipodia formation and membrane ruffles in response to growth factors such as Platelet Derived Growth Factor (PDGF) (Ridley *et. al.,* 1992). A later response is the formation of actin stress fibres; the growth factor signals through Rac1 to stimulate a Rho-dependant response (Ridley *et. al.,* 1992), a first indication of the inter-dependence/cross-talk in GTPase signalling. During lamellipodia extension, Rac1 regulates actin polymerisation by activating actin polymerising proteins including the Arp2/3 complex through WAVE proteins. Rac1 may also increase the availability of actin monomers through regulation of cofilin or by removing capping proteins (Heasman and Ridley, 2008). In hippocampal neurones, increased dynamics of dendrites and dendritic branches induced by N-Methyl-D-Aspartic acid (NMDA), is accompanied by endogenous Rac activation and inhibited by the expression of dominant negative Rac1 (Henle *et. al.,* 2006). Rac1 has also been shown to regulate growth cone collapse or expansion in response to different extracellular ligands (Jin and Strittmatter, 1997; Kuhn *et. al.*, 1999; Vastrik *et. al*., 1999; Shekarabi *et. al.,* 2005).

Cdc42 produces peripheral actin microspikes and filopodia formation in response to the growth factor bradykinin (Nobes and Hall, 1995; Kozma *et. al.*, 1995). Cdc42 is involved in yeast budding, epithelial polarity, migratory polarity and fate specification in cell division. Downstream targets for Cdc42 in the formation of filopodia include mDia (Mellor, 2010), members of the formin family and insulin receptor substrate (IRS) p53

(Govind *et. al.*, 2001; Krugmann *et. al*., 2001). LIM kinase is also activated downstream of Cdc42 and p21 Activated Kinase (PAK), which stimulates phosphorylation and inactivates cofilin (Heasman and Ridley, 2008).

1.3.4 Cell Adhesion and Rho

RhoA and ROCK are involved in the formation of focal adhesions and stress fibres leading to contractility (Dhawan and Helfman, 2004). ROCK is also regulated downstream of integrin signalling in cell migration (White *et. al.,* 2007). Integrins are a large family of cell adhesion receptors that mediate cell matrix adhesion and transduce cell signals (Hynes, 2009). The αβ1 integrins form focal adhesions comprised of a complex of proteins that associate with the cytoskeleton (Schober *et. al.,* 2007). Integrins lack enzymatic activity and therefore rely on a variety of proteins such as FAK to function (Hynes, 2002). A FAK-Src complex is important in integrin regulation of the Rho GTPases in cell adhesion (Huveneers and Danen, 2009).

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1.3.5 GTPases in Endocytosis and Vesicle Transport

Endocytosis and vesicle trafficking involves small GTPases of the Rab family, of which there are more than 60 members, localised to different membrane compartments (Pfeffer and Aivazian, 2004). Rab 5 is a marker of early endosomes, Rab7 is present in late endosomes and lysosomes and Rab11 in Golgi and recycling endosomes (Hutagalung and Novick, 2011). Rabs function through effector proteins to achieve specificity (Grosshans *et. al.,* 2006). In vesicle trafficking, Rabs can recruit as effectors GAPs and GEFs for upstream and downstream signalling (Hutagalung and Novick, 2011). Some Rab

effectors are able to recognise more than one Rab protein, such as Rabaptin5, which interacts with Rab4 and Rab5, connecting endocytosis and recycling (Vitale *et. al.*, 1998). Rho GTPase regulation of the actin cytoskeleton is also important in endocytosis. Expression of dominant active mutations of Rho and Rac in different cell types affects endocytosis of different types of receptors (Takai *et. al.*, 2001). RhoD and RhoB have been shown to localise to early and late endosomes respectively (Murphy *et. al.*, 2001; Adamson *et. al.*, 1992) further implicating the Rho GTPases as having an important role in endocytosis. Rac1 has been shown to drive endocytosis of the plasma membrane in response to ephrin A2 and sema 3A, causing growth cone collapse (Jurney *et. al.*, 2002).

Cells require vesicle transport for a variety of functions, including establishing and maintaining cell polarity, by transporting proteins to distinct cellular locations. The transport of vesicles has four different stages, vesicle formation, intracellular transport (which involves myosins, kinesins and dyneins (Mallik and Gross, 2004)), vesicle tethering and membrane fusion (involving the SNARE proteins (Chen and Scheller, 2001)). The Rab proteins and Rab effectors play a critical role in all four stages of vesicle transport, all of which are highly regulated and not fully understood (Grosshans *et. al.*, 2006). The Rho GTPases are also important in vesicle transport, and associate with the exocyst complex, an octameric complex that tethers secretory vesicles to the plasma membrane prior to fusion. The Rho GTPases are involved in cell polarity and membrane transport regulation and have been associated in playing a role in downstream vesicle fusion (Wu *et. al.*, 2008).

1.3.6 Rho GTPases in Neurones

In differentiating neurones, RhoA, Rac1 and Cdc42 have distinct and overlapping roles. Temporal and spatial differences, or GEFs and GAPs responding differently to upstream signals, provide specificity in cell function of Rho GTPases (Lowery and Van Vactor, 2009). Rho mediates ROCK activation and translocation to the membrane where they phosphorylate specific substrates in cell contractility mentioned above (Section 1.3.3/4) (Leung *et. al.*, 1996; Matsui *et. al.*, 1996). A major brain substrate of ROCK is CRMP-2, which is phosphorylated at threonine 555 (Arimura *et. al.*, 2000). CRMP-2 is involved in growth cone collapse via the sema 3A pathway (Goshima *et. al*, 1995) and neuronal polarity and axonal outgrowth (Inagaki *et. al*, 2001).

Rac1 regulates a major effector, the PAK serine/threonine kinases, of which there are six known members. PAK1 and PAK3 are highly enriched in neurones and induce the formation of dendritic spines (Zhang *et. al.*, 2005). The PAK N-terminal auto-inhibitory domain prevents the activity of the C-terminal kinase domain. Association of the auto-inhibitory domain of PAK with Rac1-GTP or Cdc42-GTP disrupts the auto-inhibition (Manser *et. al.*, 1994). Cdc42 is able to activate Rac1 and PAKs 1-3 and therefore, distinguishing between which proteins Cdc42 and Rac1 activate is problematic (Bernard *et. al.*, 1999). Rac1 also binds p35/Cdk5 a serine/threonine kinase (Nikolic *et. al.*, 1998), which is involved in neurite outgrowth during neuronal differentiation (Nikolic *et. al.*, 1996).

1.3.7 Adaptor Proteins-Nck

Nck1 and 2 are adaptor proteins containing three SH3 domains followed by a C-terminal SH2 domain. Nck couples phosphotyrosine signalling to proteins that regulate the actin cytoskeleton. Nck SH2 domain interacts with active tyrosine phosphorylated RTKs, such as PDGF receptor, epidermal growth factor (EGF) and ephrins, and the SH3 domains bind to proteins that regulate the cytoskeleton, such as, N-WASP. Nck can activate N-WASP, by binding to the polyproline region (Tomasevic *et. al.,* 2007). Rac and Cdc42 GTPases also promote Arp2/3 association with WASP family members and actin polymerization regulators of the cytoskeleton (Bladt *et. al.,* 2003).

Nck also associates with G-protein coupled receptor kinase-interacting protein 1 (GIT-1), an Arf-GAP (Frese *et. al.,* 2006). Activation of the adaptor protein Nck, results in the membrane localisation of the serine/threonine kinase PAK (Lu *et. al*., 1997; Sells *et. al*., 1997). Nck interacts with PAK1 through the second SH3 domain and regulates membrane localisation and kinase activity synergistically with Cdc42. Activated PAK1 phosphorylates LIM kinase which subsequently phosphorylates and inhibits cofilin affecting actin organisation. PAK1 also has a scaffolding function, binding the SH3 domain of a Cdc42-GEF PIX, and Rac1 (Bladt *et. al.,* 2003). Nck2 has been implicated in integrin signalling (Section 1.3.4), binding the LIM4 domain of PINCH through its third SH3 domain (Tu *et. al.,* 1998), and therefore linking integrin signalling through PAK to cytoskeleton rearrangement (Buday *et. al.,* 2002).

1.4 Axonal Guidance

Neurones are highly specialised and polarised cells, which receive and transmit signals through dendrites and an axon, which extend over long distances. During development, growing axons are directed to their targets by attractive and repellent guidance cues. The growth cone is situated at the growing tip of the axon and is a 'spread-out' and very dynamic structure, constantly lengthening and retracting filopodia in response to guidance cues.

Neuronal growth cones express cell adhesion molecules which regulate adhesion to the extracellular matrix and to other cells, enabling the movement of the axon (Chisholm and Tessier-Lavigne, 1999). Molecular guidance cues include members of the semaphorin family, ephrins, netrins, slits, neurotrophins and neurotransmitters (Mortimer *et. al.*, 2007). Molecular guidance cues can be soluble, such as class 3 semaphorins, or cell membrane associated, such as other classes of semaphorins and ephrins.

1.4.1 Semaphorin Signalling

Semaphorins are widely expressed in the nervous system, the immune system and the heart and vascular system, affecting cell migration, branching of the vascular system and immune cell regulation (Kruger et. al., 2005). In the brain, semaphorins are mainly repulsive guidance molecules, but also elicit an attractive response in the axons of developing peripheral neurones (Wong *et. al.*, 1999) and apical dendrites of cortical neurones (Polleux *et. al.*, 2000). There are eight classes overall, classes 1 and 2 are invertebrate, 3-7 are vertebrate and 8 (or V) is virally encoded. Classes 1, 4, 5 and 6 are transmembrane proteins, class 7 is tethered to membranes via a GPI anchor and class 3

(as well as 2 and V) are secreted proteins. All contain a Sema domain at the N-terminal and a PSI (plexins, semaphorins and integrins) domain, (except for viral semaphorins, that do not contain a PSI domain). Sema domains are also found in the extracellular region of plexins (sema co-receptors) and in the RTKs Met and Ron (Gherardi *et. al*., 2004). In addition to the Sema domain, plexins have three PSI domains and three IPT (Ig-like, plexins and transcription factors) domains and in their intracellular region is a GAP homology region, which is split into two by a GTPase binding domain. There are nine mammalian plexins A1-4, B1-3, C1 and D1. Neuropilins-1 and -2 are transmembrane sema receptors with short cytoplasmic tails, which form complexes with plexin-As (Negishi *et. al*., 2005). Of class 3 soluble semaphorins, sema 3A is associated with neuropilin 1, sema 3F with neuropilin 2 and sema 3C with a neuropilin 1/2 heterodimer (Chisholm and Tessier-Lavigne, 1999).

Sema 3A is the most studied, with neuropilin-1/plexin-A as its receptor complex. Rho GTPases are involved in this signalling pathway and cause a repulsive response. Growth cone collapse initiated by sema 3A signalling involves the Rho GTPase Rac1 (Jin and Strittmatter, 1997). Downstream from sema 3A ligand binding, signalling to microtubules and to actin may act simultaneously to alter the cytoskeleton and cause growth cone collapse (Goshima *et. al*., 1995; Aizawa *et. al*., 2001).

Sema 3A binds neuropilin-1, making the cytoplasmic region of plexin-A1 available for Fes/Fps binding and phosphorylation, stimulating the repulsive response (Negishi *et. al*., 2005). Fes/Fps tyrosine phosphorylates a CRMP-2/CRAM (CRMP-5) complex (Mitsui *et. al.*, 2002). CRMP-2 was identified as an essential component of sema 3A signalling (Goshima *et. al.*, 1995) and its interaction with microtubules is regulated by

phosphorylation (especially serine/threonine phosphorylation by Cdk5 and GSK3β) (Brown *et. al.,* 2004; Yoshimura *et. al.,* 2005). Cdk5 and GSK3β are also participants in sema 3A signalling (Sasaki *et. al.,* 2002; Eickholt *et. al.,* 2002) and their substrates include Tau and MAP, affecting the cytoskeleton.

Fyn tyrosine kinase and Cdk5 serine/threonine kinase form an activity dependent complex that associates with the plexin-A cytoplasmic domain in response to sema 3A stimulation, Fyn phosphorylates Cdk5 (Sasaki *et. al.* 2002). Activated Cdk5 phosphorylates CRMP-2 at serine 522 which primes for phosphorylation by the serine/threonine kinase GSK-3β at serine 518 and threonines 514 and 509. This phosphorylation cascade of CRMP-2 by Cdk5 and GSK-3β is essential for sema 3A growth cone collapse and neuronal polarity (Brown *et. al.,* 2004; Yoshimura *et. al.,* 2005). CRMP-2 binds to tubulin heterodimers promoting microtubule assembly (Fukata *et. al.,* 2002), but recently has been shown to stabilize microtubules from which it is released by phosphorylation (Lin *et. al*., 2011). Rho kinase phosphorylates CRMP-2 at threonine 555, this has been shown to reduce CRMP-2 binding of tubulin (Arimura *et. al*., 2000).

Down regulation of sema 3A signalling at the growth cone may occur by endocytosis of neuropilin-1, and the cell adhesion molecule L1 has been implicated (Castellani *et. al*., 2000). L1 is expressed along the axon shaft, but is preferentially expressed at the growth cone where it binds neuropilin-1 and is responsible for determining the attractive or repulsive response. After semaphorin binding, L1 controls endocytosis, thereby controlling the turnover of the neuropilin receptor (Castellani *et. al*., 2002). Sema 3A and

Fyn signalling has also been reported to influence dendrite branching in brain development (Morita *et. al*., 2006).

1.4.2 Ephrin-Eph Signalling

Eph-ephrin signalling regulates dendritic spine formation and is involved in many aspects of synaptogenesis, including presynaptic differentiation and neurotransmitter release (Klein, 2009). In vertebrates, the Eph family of receptors are the largest subfamily of RTKs. Eph receptors are split into two subclasses based on sequence and ligand specificity; there are nine EphAs (EphA1-8 and EphA10) and five EphBs (EphB1-4 and EphB6). Their membrane-bound ligands, ephrins are also split into two subclasses, ephrin A1-5 and ephrin B1-3. Ephrin As are attached to the membrane via a GPI anchor, whereas, ephrin Bs have transmembrane domains. Figure 1.4 shows a diagrammatic representation of Eph receptors and their ligands. Mainly ephrin A ligands signal through the EphA receptors and ephrin B ligands signal through EphB receptors, although there are some exceptions, the most well characterised being the EphA4 receptor which can bind both A and B ephrins. The large number of Eph receptors and ephrin ligands suggests, there may be some functional redundancies and/or some specific functions that are as yet unidentified (Murai and Pasquale, 2004).

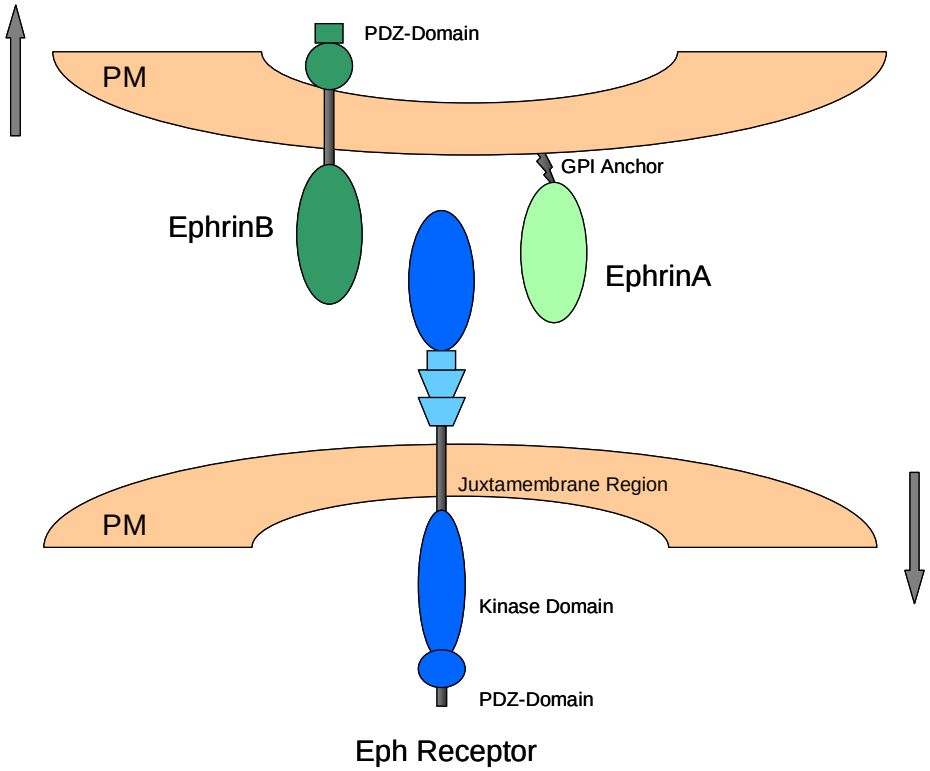


Figure 1.4: Ephrin/Eph signalling Ephrin As are GPI-linked to the plasma membrane (PM), whereas ephrinBs are transmembrane proteins. Signalling can occur in either direction, forward from the ligand to the Eph receptor, or in reverse from the receptor to the ephrins.

Although the Eph family are RTKs, they are quite unusual in the way that they function. The Eph-ephrin interaction requires a high order of ligand clustering for a signal to occur and the Eph-ephrins can signal bi-directionally; forward signalling from the ligand to the receptor, and reverse signalling from the Eph receptor to the ephrin ligand in the neighbouring cell. As Eph receptors and ephrin ligands can be expressed on the same cell surface, it is possible that *cis* interactions of the ligand and receptor on the same cell may also occur (Carvalho *et. al.*, 2006). The ephrin-Eph signalling pathway and many other RTKs, recruit phosphotyrosine binding adaptor proteins containing SH2 and/or SH3 domains, for example Nck and other proteins such as Phosphoinositide 3-Kinase (PI3K),

and signal downstream to RhoGTPases to remodel the actin cytoskeleton (Noren and Pasquale, 2004).

In forward signalling, ephrin-Eph binding triggers large clusters of both receptors and ligands on the cell surface, causing auto-phosphorylation of two conserved tyrosines in the juxtamembrane region of Eph (Goldshmit *et. al.*, 2006). The juxtamembrane domain of the Eph receptor is auto-inhibitory until tyrosine autophosphorylation occurs in response to ligand stimulation. Eph auto-phosphorylation then enhances kinase activity and provides a docking site to recruit SH2 domain containing proteins, such as SFKs (Ellis *et. al.*, 1996; Zisch *et. al.,* 1998), PLCγ and PI3K (Murai and Pasquale, 2004).

The GEF, ephexin, provides a direct functional link between EphA receptors and Rho GTPases (Shamah *et. al.*, 2001). Ephexin specifically binds EphA receptors in the absence of stimulation by ephrins. Upon activation by ephrins, EphA4 phosphorylates ephexin at tyrosine 87 and ephexin then activates RhoA and inhibits Cdc42 and Rac1, in turn, inhibiting PAK and PAK mediated events, promoting growth cone collapse (Huot, 2004). Ephrin induced growth cone collapse or retraction requires Rho and ROCK and additionally, Cdc42 and MRCK signalling pathways (Groeger and Nobes, 2007). Ephrin A5 mediated collapse signals through Rho and ROCK and Rac is down regulated (Wahl *et. al.*, 2000).

Cdk5 is involved in the ephrin A induced signalling pathway (Cheng *et. al.*, 2003). Activation of EphA4 leads to the recruitment and subsequent tyrosine phosphorylation of Cdk5, causing Cdk5 activation (Fu *et. al.*, 2007). Active Cdk5 phosphorylates two actin cytoskeleton regulators, ephexin1 and WAVE-1, leading to the loss of dendritic spines,

and it was shown that phosphorylation of ephexin1 by Cdk5 is necessary for ephrin A1-induced spine retraction (Fu and Ip, 2007).

Reverse signalling has mainly been studied in the B class of Ephs and ephrins, the A class can also participate in reverse signalling, although the ephrin As do not have a transmembrane domain. Ephrin A can trigger intracellular signalling pathway activation through association with transmembrane proteins, such as p75 neurotrophin receptor and the TrkB receptor (Lim *et. al.*, 2008; Marler *et. al.*, 2008). Ephrin B reverse signalling may use a phosphotyrosine-independent docking mechanism rather than the intrinsic kinase domain used in forward signalling (Mäkinen *et. al.*, 2005). Ephrin Bs take part in reverse signalling by recruiting PDZ-containing proteins to their C-terminal, PDZ-binding domain, and these recruit serine/threonine kinases to transmit the signal (Torres *et. al.*, 1998). Rho GTPases and Src family kinases are also involved in ephrin B reverse signalling (Egea and Kein, 2007). In neurones, ephrin B reverse signalling promotes the maturation of dendritic spines. This process involves the adaptor protein Nck2, GIT1 and β-PIX, an exchange factor for Rac (Zhang *et. al*., 2005; Segura *et. al.* 2007).

Ligand-receptor complexes are endocytosed to suppress the Eph-ephrin signalling pathway. Internalisation of ephrin B/EphB complex and consequent cell retraction is a Rac1 dependent pathway (Marston *et. al.*, 2003). A Rho-GEF Vav2, binds to the phosphorylated tyrosines in the juxtamembrane domain of activated EphA4 through its single SH2 domain and Vav2 knock out studies in mice have shown a defect in Eph endocytosis, suggesting that Vav2 plays a role. Moreover, the phosphorylation of Vav2 promotes local Rac1-dependent endocytosis, which induces an ephrin-Eph repulsive response (Cowan *et. al.*, 2005). Another possibility for Eph-ephrin signalling suppression

is the cleavage of the ephrin A ectodomain by A-Disintegrin-And-Metalloprotease (ADAM) 10. ADAM10 only cleaves ephrin As on the opposite cell surface (Janes *et. al.*, 2005). Alternatively, to deactivate Eph-ephrin signalling, the Eph receptor juxtamembrane tyrosines can be dephosphorylated by a phosphotyrosine specific protein phosphatase (PTP). Protein tyrosine phosphatase receptor type O (Ptpro) has been suggested for this as it is a PTP that dephosphorylates EphA and B receptors (Shintani *et. al.*, 2006).

Studies using knock out mice have shown that when EphA4 or ephrin B3 are absent, mice produce axons with guidance defects that inappropriately cross the spinal cord midline, resulting in mice with a rabbit-like hopping gate (Dottori *et. al*., 1998; Kullander *et. al.*, 2001) showing an essential developmental role of ephrin B3/EphA4 signalling in correct axonal positioning in motor pathways.

1.4.3 Netrins

Netrins are soluble guidance factors which interact with cell membranes through negatively charged proteoglycans, and can act as an attractant or a repellent to growth cones depending upon which receptor they bind. Netrins signalling through a transmembrane receptor of the Deleted in Colorectal Cancer (DCC) family, with four immunoglobulin domains and six fibronectin type III repeats, cause an attractive response. A repulsive response occurs when netrins signal through transmembrane receptor of the UNC-5 family, containing two immunoglobulin domains and two thrombospondin repeats (Chisholm and Tessier-Lavigne, 1999).

1.4.4 Slits

Slits are large proteins containing leucine rich and EGF-like repeats. Much of the work on the Slit family of proteins has been carried out in *Drosophila*. Slit proteins are midline repellents, which signal through a transmembrane receptor roundabout (Robo), they ensure that axons cross the midline only once. Axons expressing Robo are repelled by Slit at the midline, this works by Commissureless (Comm) keeping Robo down-regulated by acting as an endosomal sorting receptor (Keleman *et. al.*, 2002) until the axon crosses the midline. Robo is then up-regulated thereby preventing the axon from crossing back (Chilton, 2006). No Comm has been found in vertebrates, however, vertebrates contain a third Robo receptor (rig-1/Robo 3) which appears to have the same function as Comm (Allen and Chilton, 2009).

1.5 Chimaerins

1.5.1 Chimaerin Genes

Chimaerin was first isolated as a novel neuronally-expressed sequence from a human brain cDNA library, related to PKC and BCR (Hall *et. al.*, 1990). Chimaerins contain a cysteine rich, phospholipid-dependent phorbol ester binding C1 domain and were the first non-PKC phorbol ester receptors described (Ahmed *et. al.*, 1990). There are two genes for chimaerin, α and β. The α- (n-) chimaerin gene (*CHN1*) maps to chromosome locus 2q31.1 and the β-chimaerin gene (*CHN2*) to chromosome 7p15.3 (Hall *et. al.*, 1993; Leung *et. al.*, 1993; http://www.ncbi. nlm.nih.gov/gene). Each encodes two major splice variants, α1-, β1- and α2-, β2- (Figure 1.5).

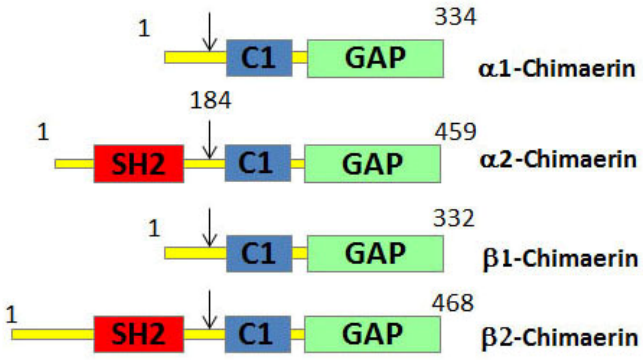


Figure 1.5: Chimaerin splice variants Two chimaerin genes, α and β, each encode two isoforms α1- and α2- and β1- and β2-chimaerin respectively. Chimaerins α2- and β2- contain an SH2 domain in addition to the C1 and GAP domains present in all chimaerins. Amino acid numbers are shown and the position corresponding to the conserved alternative splice site is indicated (α2-chimaerin 184).

The protein sequences of α2-chimaerin and β2-chimaerin SH2, C1 and GAP domains are highly similar, with 81.8%, 85.5% and 75% identity, respectively and there is greater sequence divergence in the SH2-C1 linker regions (39.1% identity) and N-terminals (66.7% identity). Between species α2-chimaerin is highly conserved; at the protein level sequence similarities are mouse 96%, chicken 95% and zebra fish 86.7% compared to human α2-chimaerin.

The rat embryonic nervous system expresses high levels of α2-chimaerin mRNA, whereas, α1-chimaerin mRNA is only detectable after embryonic day 16.5 (Hall *et. al.*, 2001) and increases in expression during development (Lim *et. al.*, 1992). Adult rat neurones express α2-chimaerin mRNA in specific brain regions; the highest being the anterior olfactory nucleus, hippocampus, cortex and entorhinal cortex (Hall *et. al.*, 2001). Additionally, α2-chimaerin is expressed in rat testis (Hall *et. al.*, 1993). β1-Chimaerin is mainly expressed in testis (with a different expression pattern to α2-chimaerin), and is associated with spermatogenesis (Leung *et. al.*, 1994). β2-Chimaerin is highly expressed in cerebellum and is also found in T-lymphocytes (Leung *et. al.*, 1994; Siliceo *et. al.*, 2006). α2-Chimaerin expressed in mammalian cells is predominantly soluble, with only a fraction being membrane associated whilst α1-chimaerin is almost entirely membrane and cytoskeleton bound. A point mutation in the SH2 domain of α2-chimaerin was sufficient to produce an α1-chimaerin-like phenotype (Hall *et. al.*, 2001).

1.5.2 Chimaerins as RacGAPs

Chimaerin was identified as a RhoGAP for Rac1 (Diekmann *et. al.*, 1991; Manser *et. al.*, 1994), while its GAP domain is highly related to BCR C-terminal domain (Hall *et. al.*, 1990; Ahmed *et. al.*, 1994). Similar GAP domains are present in the large RhoGAP superfamily (Bernards and Settleman, 2004). Chimaerins as RacGAPs play an important role in limiting/inhibiting Rac-dependent actin reorganisation and cell morphology. Early work in mammalian cells indicated that chimaerin expression can act in conjunction with Rac1 and Cdc42 to stimulate actin rearrangement, forming lamellipodia and filopodia, independently of GAP activity. This suggested that α-chimaerin may have morphological

functions in association with other proteins in addition to down regulating GTPases (Kozma *et. al.,* 1996). In N1E-115 neuroblastoma cells, permanent expression of α2-chimaerin generated neuritis, whereas permanent expression of α1-chimaerin produced microspikes and F-actin clusters but very few neurites (Hall *et. al.,* 2001). The different morphology possibly reflects the differences in GAP activity and protein interactions of the two isoforms. The RacGAP activity of chimaerins is sensitive to phorbol ester/DAG, phosphatidylserine and phosphatidic acid via the C1 domain (Ahmed *et. al.,* 1993; Ahmed *et. al.,* 1994; Caloca *et. al.,* 2003).

1.5.3 Chimaerin C1 Domain

Chimaerin C1 domain has approximately 40% homology with PKC isoenzyme C1 domains and contains the necessary amino acid residues for phorbol ester binding and the hydrophobic residues required for membrane insertion (Caloca *et. al.,* 2001). In *in vitro* studies, chimaerins, like PKC, bind DAG and phorbol esters with high affinity in a phosphatidylserine-dependent manner (Ahmed *et. al.,* 1990; Caloca *et. al.,* 1997; Areces *et. al.,* 1994). Chimaerin C1 domain is important in regulating the localisation of chimaerins, as phorbol ester treatment induces translocation of chimaerins from the cytosol to membranes (Caloca *et. al.,* 1999; Caloca *et. al.,* 2001). The C1 domain mutation of β2-chimaerin C246 is sufficient to block phorbol ester/DAG binding and membrane translocation (Caloca *et. al.,* 1999). Mutants with reduced affinity for phorbol esters, including P216 in α2-chimaerin (Colón-González *et. al.,* 2008) and F215 in β2-chimaerin (Siliceo *et. al.,* 2006), have also been utilized to inhibit C1 domain function.

1.5.4 Chimaerin Structure

The crystal structure of β2-chimaerin was resolved by Canagarajah *et. al.* in 2004, which revealed an inactive, closed conformation (Figure 1.6). The C1 domain has many intramolecular contacts throughout the protein. A helix of eight residues within the N-terminal of β2-chimaerin lies against the membrane binding site and conceals the phorbol ester binding groove. The C1 domain also has contacts with the SH2 domain, the GAP domain and the SH2-C1 inter-domain linker and in all approximately 40% of the C1 domain is buried. The SH2 domain appears to have a phosphotyrosine binding pocket of three arginine residues and a glutamine residue, however, unusually one of the arginine residue side chains is rotated away from the binding site. The Rac1 interacting surface of the C-terminal GAP domain is concealed by two proline residues in the N-terminal of β2-chimaerin (Canagarajah *et. al.*, 2004). This demonstrated a closed conformation of inactive chimaerin with hidden binding sites. Binding of phospholipids at the C1 domain involves dissociation of the tightly wound structure of chimaerin, unblocking the active site and activating chimaerin (Canagarajah *et. al.*, 2004).

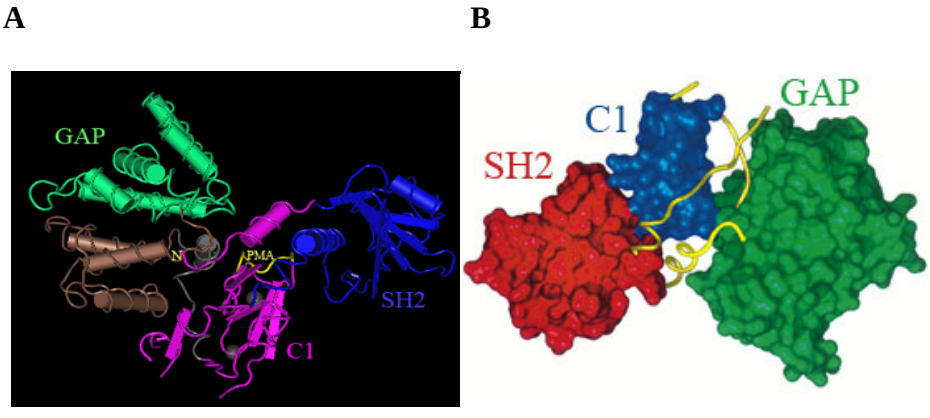


Figure 1.6 Structure of α2-chimaerin (**A:** α2-Chimaerin structure from http://www.ncbi.nlm.nih.gov/structure/, **B:** β2-Chimaerin structure adapted from Caraganajah *et. al.*, 2004). **A:** Structure of α2-chimaerin demonstrating the

partially buried nature of the C1 domain. The C-terminal GAP domain is shown in green and brown, the C1 domain is purple, with the co-ordinated zinc shown as grey spheres, and the SH2 domain is blue. The extreme N-terminal (yellow N) and SH2-C1 linker contact the other domains. The phorbol ester binding site is shown in yellow (Phorbol Myristate Acetate (PMA)). **B:** Space filling diagram of β2-chimaerin also showing the obscured nature of the C1 domain. The linker regions and the N-terminal are shown in yellow.

1.5.5 Chimaerin Regulation – C1 Domain

Chimaerins are more resistant to membrane translocation by phorbol esters than PKC (Caloca *et. al.,* 1999; Caloca *et. al.,* 2001; Colón-González *et. al.,* 2008), possibly due to C1 domain occlusion by intramolecular interactions (Caraganajah *et. al.,* 2004; Colón-González *et. al.,* 2008). This has also been demonstrated for MRCK (Tan *et. al.,* 2001), where MRCK C1 domains are more resistant to DAG than PKC, similar to chimaerins (Choi *et. al.,* 2008). Structural differences in the binding cleft or membrane binding regions of various C1 domains may also play a role in DAG signalling (Colón-González and Kazanietz, 2006).

Interestingly it was reported that a series of β2-chimaerin mutants (L28A, I130A and I359A) that disrupt intramolecular interactions show enhanced translocation, increased binding to acid phospholipid vesicles and increased GAP activity (Caraganajah *et. al.,* 2004; Sosa *et. al.,* 2009). Similarly, N-terminal single point mutations of L20A and I122A in α2-chimaerin, which were predicted to disrupt intramolecular interactions, were more sensitive to phorbol ester and translocation, and I122A showed enhanced RacGAP activity compared to wild type α2-chimaerin (Colón-González *et. al.,* 2008).

Response to different phorbol esters affects the location of β2-chimaerin and PKCα, suggesting that DAG signalling regulates their cellular location. This may also involve protein-protein interaction mechanisms (Caloca *et. al.,* 2001). Chimaerin isoforms may

localise to the golgi apparatus through their C1 domain interaction with the endoplasmic reticulum and golgi protein P23/Tmp21. This indicates that the C1 domain interacts with both proteins and lipids (Wang and Kazanietz, 2002; Wang and Kazanietz, 2010). Phorbol esters also promote the association of β2-chimaerin with Rac1 (Caloca *et. al.,* 2001) and increase stability of α1-chimaerin (Marland *et. al.,* 2011).

1.5.6 SH2 Domain Interactions

Chimaerins α2- and β2- contain an N-terminal SH2 domain in addition to C1 and GAP domains (Hall *et. al.,* 1993; Leung *et. al.,* 1994). SH2 domains specifically recognise phosphotyrosine containing proteins and are present in a wide spectrum of proteins including GTPase regulatory proteins like vav and RasGAP, adaptor proteins such as Nck and Grb2, and the phospholipid signalling protein PLCγ (Pawson *et. al.,* 2001), as well as SFKs. The SH2 domains of α2- and β2-chimaerin are atypical in that they contain a glutamic acid residue in the place of the conserved tryptophan residue at the N-terminal end of SH2 domains. This substitution in mutated Src SH2 resulted in altered phosphotyrosine binding (Bibbins *et. al.,* 1993), possibly indicating that chimaerin phosphotyrosine binding may be different to that of conventional SH2 domains. However, the essential arginine residues required for phosphotyrosine-binding are conserved in chimaerins (Hall *et. al.,* 1993; Leung *et. al.,* 1994). The role of α2- and β2-chimaerin SH2 domains is still unclear, and a phosphotyrosine-dependent target has not been unequivocally identified. Although α2-chimaerin interacts with the RTK EphA4, whether this is a phosphotyrosine-dependent SH2 interaction is not established (see below). The α2-chimaerin SH2 domain interacts with other proteins, such as CRMP-2, however, the interaction may not require phosphotyrosine binding (Brown *et. al.,* 2004).

Over-expression of mutations in the SH2 domain of conserved arginines (R73 and R56), which abolish phosphotyrosine interactions (Waksman *et. al.,* 1993; Hall *et. al.,* 2001) did not prevent CRMP-2 binding to the α2-chimaerin SH2 domain (Brown *et. al.,* 2004).

The roles of chimaerin phosphorylation are currently under investigation and studies have been emerging over the last few years, during the course of this project (Kai *et. al.,* 2007; Siliceo and Mérida, 2009; Griner *et. al.,* 2010). Over-expressed β2-chimaerin in COS cells was reported to be phosphorylated by PKCδ at serine169 when stimulated by PMA or EGF. In addition, endogenous β2-chimaerin was phosphorylated in cerebellar granule neurones in response to BDNF (Griner *et. al.,* 2010). An S169A mutant of β2-chimaerin was reported to be more sensitive to C1 mediated translocation from the cytosol to the membrane, with an enhanced effect on Rac-GTP in EGF treated COS cells (without altering intrinsic *in vitro* Rac-GAP activity). S196 phosphorylated β2-chimaerin detected by a phospho-antibody was soluble and not in the particulate fraction (membrane associated). Phosphorylation of β2-chimaerin S169 by PKCδ leads to its retention in the cytosol and restricts relocalisation and activation (Griner *et. al.,* 2010). S169 in β2-chimaerin lies in the SH2-C1 linker region, which in contrast to the C1 domain is not well conserved between α and β-chimaerins. In fact, the sequence divergence in this region could underlie differences in the regulation of α2- and β2-chimaerin.

Tyrosine phosphorylation of β2-chimaerin Y21 in EGF-treated COS cells by SFKs was reported to have negative regulatory effects on its RacGAP activity (Kai *et. al.,* 2007). Y21F (but not Y153F, a mutant that did not show reduced phosphorylation) showed significantly increased GAP activity towards RacGTP (Kai *et. al.,* 2007). However,

tyrosine phosphorylation of β2-chimaerin by Lck in T-cells was mapped to Y153 which negatively regulated β2-chimaerin membrane stabilisation, leading to its decreased RacGAP activity (Siliceo and Mérida, 2009).

1.5.7 Chimaerins in Cell Signalling

β2-Chimaerin can function downstream of EGFR, which activates pathways leading to DAG generation and Rac activation. Fluorescent resonance energy transfer studies were used to show that translocation of β2-chimaerin mediated by EGF causes its association with Rac1 at the plasma membrane (Wang *et. al.*, 2006). β2-Chimaerin has also been implicated as a tumour suppressor in breast cancer, inhibiting cancer cell proliferation and metastasis (Menna *et. al.*, 2003; Yang *et. al.*, 2005). In addition, chimaerins have a role in the immune system, where their catalytic activity is important in T-cell receptor inhibition and also in inhibition of Vav1-dependant stimulation of the transcription factor NF-AT (Caloca *et. al.*, 2008), which is vital for T-cell activation and differentiation (Serfling *et. al.*, 2000). β2-Chimaerin has also been shown to be important in regulating adhesion and chemotaxis of T cells and can localise at the immune synapse (Siliceo *et. al.*, 2006).

α2-Chimaerin is implicated in Sema 3A growth cone collapse through Rac1 (as described in section 1.4.1), with its SH2 and GAP domains being essential (Brown *et. al.*, 2004). Activation of α2-chimaerin by PMA induces growth cone collapse and activated α2-chimaerin interacts with CRMP-2 through its SH2 domain, and p35/Cdk5 via the GAP domain (Brown *et. al.*, 2004; Qi *et. al.*, 2004). CRMP-2 phosphorylation by Cdk5, which primes for phosphorylation by GSK3β, is required for growth cone collapse (Brown *et.*

al., 2004; Uchida *et. al.,* 2005). It was demonstrated that both α2-chimaerin and CRMP-2 can bind the sema 3A co-receptor neuropilin1/plexin-A1 (Brown *et. al.,* 2004), further implicating them in this pathway.

During the course of this study, EphA4 axonal growth cone collapse was shown to involve α2-chimaerin (Wegmeyer, *et. al.*, 2007; Beg *et. al.,* 2007; Iwasato *et. al.,* 2007; Shi *et. al.,* 2007). Chimaerin gene deletion mutant mice display similar phenotypes to that of ephrin $B3^{-/-}$ (Yokoyama *et. al.,* 2001; Kullander *et. al.,* 2001) and $EphA4^{-/-}$ mice (Dottori *et. al.,* 1998). Axon development is inappropriately targeted, resulting in defects in the spinal cord central pattern generator and locomotor function (Wegmeyer, *et. al.*, 2007; Beg *et. al.,* 2007; Iwasato *et. al.,* 2007).

EphA4 can bind to α2-chimaerin and mediates ephrin A1/EphA4 and ephrin B3/EphA4 forward signalling. Active EphA4 stimulated increased tyrosine phosphorylation of α2-chimaerin and was reported to enhance α2-chimaerin's GAP activity for Rac1, inactivating Rac1 and inhibiting growth cone extension in motor circuit formation (Iwasato *et. al.,* 2007; Shi *et. al.,* 2007). However, Src-family kinase activity (inhibited by PP2) was also required in the pathway (Shi *et. al.,* 2007). Whether chimaerin binds EphA4 via a tyrosine phosphorylation dependent interaction is not entirely clear. Two groups reported that there were two EphA4 binding sites in chimaerin (Beg *et. al.,* 2007; Wegmeyer, *et. al.,* 2007), and a further study reported an EphA4 interaction with the C-terminal of both α1- and α2-chimaerin (Iwasato *et. al.,* 2007). β2-Chimaerin has also been shown to bind to EphA4 and EphA2 and inactivate Rac1 following ephrin A1 treatment (Wegmeyer, *et. al.,* 2007; Iwasato *et. al.,* 2007; Shi *et. al.,* 2007; Takeuchi *et.*

al., 2009). Ephrin A1 induces suppression of cell migration and knock down of β2-chimaerin abolished this effect (Takeuchi *et. al.,* 2009). EphA4 has been shown via peptide array to directly phosphorylate α2-chimaerin *in vitro* at tyrosines 21, 125, 143, 333 and 357 (Warner *et. al.,* 2008).

The adaptor proteins Nck1 and Nck2, were also shown to interact with Eph receptors, including EphA4 (Holland *et. al.,* 1997; Bisson *et. al.,* 2007). Furthermore, α2-chimaerin (but not α1-chimaerin) was shown to bind Nck1 and Nck2 in a phosphotyrosine independent manner. This interaction requires the α2-chimaerin SH2 domain containing N-terminal (amino acids 1-183) and one of the three SH3 domains of Nck (Wegmeyer *et. al.,* 2007). These studies suggest a role for Rac1, chimaerins, Nck proteins and tyrosine phosphorylation in ephrin/EphA4 growth cone collapse.

1.5.8 Other Functions of α2-Chimaerin

Mutations in the human *CHN1* gene are involved in Duane's retraction syndrome, a congenital eye movement disorder caused by an inability of ocular motor neurones to effectively innervate their target muscles (Miyake *et. al.,* 2008). These mutations in the coding region, including the recently reported Y148F mutation (Miyake *et. al.,* 2011), increased the RacGAP activity *in vitro* and are suggested to influence chimaerin structure as described (Caraganajah *et. al.,* 2004; Colón-González *et. al.,* 2008; Sosa *et. al.,* 2009).

In addition to its vital role in the development of ocular motor axons and locomotor circuits, α2-chimaerin is involved in cell migration. Knock down of the zebra fish *CHN1* gene homologue resulted in severe abnormalities, with defects in cell adhesion and

migration, indicating a key role for α2-(/β2-) chimaerin in early development (Leskow *et. al.*, 2006).

Recently, α2-chimaerin in mice was shown to control neuronal migration and functioning of the cerebral cortex with the involvement of CRMP-2 (Ip *et. al.*, 2011). *In utero* suppression of α2-chimaerin arrested neuronal migration at the multipolar stage, with an accumulation of neurones in the subcortical region. As a result, mice with migration defects showed an imbalance between excitation and inhibition in local cortical circuitry and evidence of susceptibility to epileptic seizures (Ip *et. al.*, 2011).

α1-Chimaerin has a role in the regulation of synaptic activity in adult neurones. The C-terminal interacts with the NR2A subunit of NMDA receptors and over expression of α1-chimarin in hippocampal neurones inhibits spine formation and prunes existing spines (Van de Ven *et. al.*, 2005). Stimulation of phospholipase Cβ in hippocampal neurones recruits α1-chimaerin to the membrane and pruning of dendrites on α1-chimaerin expression required both DAG binding and an active GAP domain (Buttery *et. al.*, 2006).

Literature points to a vital role for chimaerins in the developing nervous system and adult brain, in diverse signalling pathways. However, the proteins interacting with chimaerin in axonal guidance and its regulation in these signalling pathways have not yet been determined.

1.6 Aims of This Study

The aim of this study is to investigate α2-chimaerin and interacting partners in neuronal signalling pathways. As described above, α2-chimaerin can be activated by phorbol esters/DAG, but also contains an SH2 domain which indicated probable participation in phosphotyrosine regulation. Chimaerin is tightly regulated by its auto-inhibited structure and a key question is how α2-chimaerin is activated in neuronal guidance pathways, could it be via DAG or phosphotyrosine signalling or both? This investigation aims to discover the tyrosine kinases and residues involved in the tyrosine phosphorylation of α2-chimaerin. It will also try to establish what effects tyrosine phosphorylation of α2-chimaerin may have on neuronal development and in the sema 3A and ephrin A1/EphA4 pathways in which α2-chimaerin is now known to play a role.

The second part of this study aims to investigate new and established α2-chimaerin partner proteins in these pathways, particularly SH2 domain associated partners. Novel interacting partners have emerged during the course of this study, some discovered in our laboratory by a yeast two-hybrid screen. As a direct result of this study (and others' work), α2-chimaerin has been shown to interact with a receptor, a tyrosine kinase and an adaptor protein, as well as a novel partner for α2-chimaerin involved in protein trafficking and degradation. How α2-chimaerin interacts with these partners and what functions may occur as a result of the interaction will be examined.

Ultimately the study aims to bring a greater understanding of the signalling pathways that involve α2-chimaerin and its interacting partners.

Chapter 2.0:

Materials and Methods

2.1 Materials

2.1.1 General Laboratory Reagents

Water was obtained from an ELGA Maxima SC purification system, which was autoclaved to sterilise before use. General laboratory chemicals were obtained from Sigma-Aldrich or BDH.

2.1.2 Oligomer Synthesis and cDNA Sequencing Service

Oligonucleotides were custom synthesized by Sigma-Aldrich and cDNA sequencing was carried out by Cogenics.

2.1.3 Reagents for DNA Methods

Agarose was obtained from Sigma. 50x Tris-acetate-EDTA (TAE) was from Millipore. DNA restriction enzymes were from Invitrogen, Stratagene, Promega or New England Biolabs. The DNA markers Hae III-digested φx174 and Hind III-digested bacteriophage were from Invitrogen. The site-directed mutagenesis kit was from Stratagene. Mini and Maxi prep DNA kits for DNA purification were from Qiagen.

2.1.4 Reagents for Bacterial Methods

E. coli XL1-blue $CaCl_2$ competent cells were obtained from Stratagene. Tryptone was from BD Biosciences. Yeast extract and agarose were from DIFCO.

2.1.5 cDNA Constructs

Plexin-A1 and Neuropilin 1 vectors, pcDNA3, were kindly provided by S. M. Strittmatter (Yale, USA). Fyn and K299M Fyn, pCMV5, vectors were kindly provided by M. Resh, (Sloan-Kettering, Hong Kong). EphA4 and Kinase Dead EphA4, pcDNA3, were kindly provided by N. Y. Ip (Hong Kong). Mammalian vectors used were pXJ40 with GFP, Flag and HA tags adjacent to the polylinker (E. Manser, GSK-IMCB Singapore), pXJ40-8xHIS-precission-SBP and pBIOTIN-8HIS (pGEX-T4). PsiSTRIKE vectors with GFP, or including Neomycin or Hygromycin resistance genes were from Promega.

2.1.6 Reagents for Protein Biochemistry Methods

Triton X-100 and β-mercaptoethanol were from BDH. Inhibitors DTT, sodium vanadate, PMSF and sodium fluoride were from Sigma. Protease inhibitor cocktail tablets were obtained from Roche. FLAG and HA conjugated agarose beads were from Sigma. TEMED was from Bio-Rad. Bis-acrylamide was from Severn Biotech. SeeBlue plus2 Pre-Stained Standard Protein markers were from Invitrogen. PVDF was from Perkin-Elmer. ECL hyperfilm and ECL solutions were from Amersham Healthcare. Developer and fixer solutions were from Kodak.

2.1.7 Reagents for Cell Biology Methods

DMEM, OptiMEM, Neurobasal Medium, B-27, Hanks Buffered Saline Solution, trypsin, antibiotic/antimycotic and Foetal Bovine Serum (FBS) and for transfections Lipofectamine and Lipofectamine 2000, were all obtained from Invitrogen. G418 was from Calbiochem. Disposable plastic-ware supplies were from Greiner. Nucleofector and

solution for electroporation of primary cells were from Amaxa. Slides and coverslips were from BDH. Laminin was obtained from ICN, Poly-$_D$-Lysine was from Sigma. Bovine Serum Albumin was from Jackson Laboratories. TRITC Phalloidin was from Sigma. Slide mountant was from DAKO.

2.1.8 Antibodies

Antibody	Dilution for immunoblotting	Dilution for cell immunostaining	Donor Animal	Company
Primary Antibodies				
α2-Chimaerin	1:2000	1:100	Rabbit	In House
FLAG	1:2000	1:100	Rabbit	Sigma
HA	1:2000		Rabbit	Bethyl Laboratories
Fyn	1:250		Mouse	R and D Systems
EphA4	1:200		Rabbbit	Santa Cruz
4G10	1:1000		Mouse	Upstate/Millipore
PY20	1:1000		Mouse	Signal Transduction
Vps28	1:250	1:20	Mouse	In House
Nck1	1:1000		Rabbit	Epitomics
Tau		1:100	Mouse	Chemicon
Secondary Antibodies				
Mouse HRP	1:1000		Rabbit	DAKO
Rabbit HRP	1:1000		Swine	DAKO
Trueblot HRP	1:1000		Mouse	eBioscience
Trueblot HRP	1:1000		Rabbit	eBioscience
Rabbit Cy5		1:100	Donkey	Jackson Laboratories
Mouse FITC		1:100	Donkey	Jackson Laboratories

Table 2.1: Table of Antibodies Table of antibodies, dilutions used for immunoblotting and immunostaining and supplier.

2.2 Methods

2.3 Bacterial Methods

2.3.1 Solutions for Bacterial Growth

- **LB Media-** 1 % Tryptone, 1 % NaCl, 0.5 % Yeast Extract, in ddH_2O, sterilised by autoclave.
- **LB Agar-** 1.5 % agarose in LB media, sterilised by autoclave.
- **Ampicillin-** was added at 100 µg/ml prior to use when antibiotic selection was required.

2.3.2 Production of $CaCl_2$ Competent Bacteria

A single *E. coli* XL-1 Blue competent cell colony was picked from an agar plate using a sterile toothpick and grown in 100 ml of LB media at 37 °C for approximately 3 hours until the absorbance at 600 nm measured 0.35. The bacteria were cooled on ice for 10 minutes, pelleted at 2,700 g for 10 minutes at 4 °C and the supernatant removed. The pellet was resuspended in 30 ml of ice-cold 50 mM $CaCl_2$ for 5-10 minutes, pelleted at 2,700 g for 10 minutes at 4 °C and the supernatant removed. The pellet was resuspended in 2 ml of 15 % glycerol in 50 mM $CaCl_2$ and aliquots frozen at -80 °C.

2.3.3 Transformation of Competent Bacteria

Competent XL-1 Blue or $CaCl_2$ (as described above) competent cells were thawed from -80 °C on ice. For commercial XL-1 Blue cells only, 1.7µl of β-mercaptoethanol was added to 100µl of bacteria and incubated on ice for 10 minutes. For transformation, 5 µg

of DNA was added to 100µl of bacteria, for re-transformation 1 µg of DNA was added to 10-50 µl of bacteria and this was incubated on ice for 30 minutes. The bacteria were heat shocked by heating them to 42 ^{o}C for 30 or 90 seconds, for commercial XL-1 Blue and $CaCl_2$ (described above) cells respectively and returned to ice for 2 minutes. For transformations (but not re-transformations) 1 ml of LB media was added to the bacteria for 1 hour, agitating at 37 ^{o}C. The bacteria were pelleted in a bench top centrifuge, 900 µl of the supernatant was removed, and the pellet re-suspended in the remaining 100µl of LB media. The bacteria were streaked onto an agar plate containing ampicillin and incubated overnight at 37 ^{o}C.

2.3.4 Growing Bacterial Cultures for DNA Preparation

Using a sterile toothpick, a single bacterial colony was taken off the agar plate and placed into 5 ml for mini-preparations or 250 ml for maxi-preparations of LB media supplemented with 100 µg/ml ampicillin. This was incubated overnight at 37 ^{o}C with agitation. The culture was centrifuged at 3,000 rpm for 10 minutes at 4 ^{o}C. The bacterial pellets for maxi-preparation only were washed by re-suspention in 50 ml of PBS and transferred to 50 ml falcon tubes, which were centrifuged at 3,000 rpm for 10 minutes at 4 ^{o}C. Supernatants were removed and the bacterial pellets were ready for DNA extraction, or stored at -20 ^{o}C and the DNA extracted at a later date.

2.3.5 Isolation of Plasmid DNA

Plasmid DNA was extracted from the bacterial pellets using Mini or Maxi kits from Qiagen as directed by the manufacturer's instructions. Briefly, the bacterial pellets were re-suspended and then lysed, the lysate was passed through a filter to remove genomic

DNA and cellular debris. The plasmid DNA was passed through a Qiagen column which it bound, the DNA was washed and then eluted. Despite the kit insert indicating DNA clean up should no longer be necessary, it was found in our laboratory that the stability of DNA was enhanced and therefore DNA was cleaned up by phenol chloroform extraction and ethanol precipitation (See section 2.4.2).

2.3.6 Quantification of DNA Quality and Concentration

10 µl of DNA was added to 1 ml of EB buffer (10 mM Tris/HCl, pH 8.5, Qiagen) and mixed well. The absorption of this solution was then measured by spectrophotometer at two wavelengths, 260 nm (DNA absorption peak) and 280 nm (protein absorption peak), against a blank sample of EB buffer. A ratio of the A260:A280 gave an indication of the samples purity, with a 1.8 reading considered desirable. DNA concentration was determined by the following equation (derived from an absorbance at 260 nm of 1 being equal to ~50 µg/ml of DNA in TE buffer (Sambrook and Russell, 2001));

DNA concentration (µg/ml) = Absorbance at 260 nm x 50 (the extinction coefficient) x 1/1000 (dilution factor) x 1 (the path length).

2.4 DNA Methods

2.4.1 DNA Solutions

- **6x DNA Gel Loading Buffer-** 30% glycerol**,** 0.25% Bromophenol blue**,** in 1x Tris-Acetate-EDTA (TAE) Buffer.
- **1% Agarose Gel-** 1 g Agarose, 1x TAE Buffer 100 ml, warmed in microwave until the agarose dissolved, when cool, 6 µg of EtBr was added and the gel was poured and left to set.
- **TE Buffer-** 10 mM Tris-Cl pH 7.5, 1 mM EDTA in ddH_2O.

2.4.2 Phenol/Chloroform Extraction and Ethanol Precipitation

An equal volume of phenol chloroform was added to DNA and vortexed until white, the mix was centrifuged at 14,000 rpm for 4 minutes. The top aqueous layer (containing the DNA) was carefully removed so as not to disturb the interface. The above step was then repeated. Two volumes of 100 % ethanol and 0.1 volumes of 3 M Na Acetate pH 5.2 was added to the DNA and mixed gently. This was placed on dry ice for 5-10 minutes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was removed and the pellet washed in 200 µl of 70 % ethanol and centrifuged at 14,000 rpm for 5 minutes. All liquid was removed and the pellet dried. The pellet was resuspended in an appropriate amount of TE buffer.

2.4.3 DNA Electrophoresis

1 % agarose gels were placed into an electrophoresis tank and covered with 1x TAE buffer. DNA markers were loaded to give size indication and DNA samples were

prepared by adding 6x loading buffer and then loaded into the wells. The gel was run at 100 Volts for 40-50 minutes. DNA was visualized via an ultra violet light box and pictures were taken using a Kodak EDAS 290 camera and Kodak Molecular Imaging Software.

2.4.4 Polymerase Chain Reaction (PCR)

PCR was used for site directed mutagenesis reactions and to amplify cDNA fragments to be cloned into different vectors. PCR reactions were set up as follows;

For Amplification;	For Site Directed Mutagenesis;
5 µl 10x Reaction Buffer	5 µl 10x Reaction Buffer
3 µl $MgCl_2$	10 ng Template DNA
125 ng Primer 1	125 ng Primer 1
125 ng Primer 2	125 ng Primer 2
1 µl of dNTP Mix	1 µl of dNTP Mix
0.5 µg Template DNA	3 µl QuikSolution
Made up to 50 µl with ddH_2O	Made up to 50 µl with ddH_2O
0.5 µl Taq Polymerase (5µg/µl)	1 µl *PfuTurbo* DNA polymerase

Samples were mixed and placed into the thermal cycler for PCR with a program set as follows;

	94°C	1 minute	Initialisation
28 Cycles	94°C	45 seconds	Denaturation
	50-60 °C	30 seconds	Annealing (Dependent on sequence)
	72°C	2 minutes	Elongation
	72°C	5 minutes	Final Elongation
	7°C	Indefinitely	

A sample of 10 µl was checked for product by DNA gel electrophoresis.

2.4.5 Site Directed Mutagenesis

For all site directed mutagenesis, the QuikChange XL Site-Directed Mutagenesis Kit from Stratagene was used. Several sites were mutated in α2-chimaerin. Cysteine 239 was mutated to abolish DAG binding as published for β2-chimaerin (Canagarajah *et. al*, 2004). Previous members of the laboratory had shown that α2-chimaerin can be tyrosine phosphorylated *In Vitro* towards the N-terminal region. A phosphorylation prediction website NETPhos was used to find the most likely tyrosine sites and the highest scoring tyrosines. These included Y70, 143, 148, 157 and 303. Tyrosine 303 was chosen to be mutated as it resides next to the essential arginine residue for GAP activity. Once the sites had been chosen, oligos were designed to incorporate the change of amino acid. Cysteine was mutated to alanine to prevent DAG binding and tyrosines were mutated to phenylalanine so that they can no longer be phosphorylated. The designed oligos are shown below;

- Wild type: 5'CAGGGAGTGAAATGTGCAGATTGTGGTTTGAATGTTCATAAGCAG 3'

C239A Primer 1: 5' CAGGGAGTGAAATGTGCAGATGCTGGTTTGAATGTTCATAAGCAG 3'
C239A Primer 2: 5' CTGCTTATGAACATTCAAACCAGCATCTGCACATTTCACTCCCTG 3'

- Wild Type: 5' GGTCTTAATTCTGAAGGACTATACCGAGTATCAGGATTTAGTGAC 3'

Y303F Primer 1: 5' GGTCTTAATTCTGAAGGACTATTCCGAGTATCAGGATTTAGTGAC 3'
Y303F Primer 2: 5' GTCACTAAATCCTGATACTCGGAATAGTCCTTCAGAATTAAGACC 3'

- Wild Type: 5' GTGGCTGAGGGGAGCTACCTCATCCGGGAGAGC 3'

Y70F Primer 1: 5' GTGGCTGAGGGGAGCTTCCTCATCCGGGAGAGC 3'
Y70F Primer 1: 5' GCTCTCCCGGATGAGGAAGCTCCCCTCAGCCAC 3'

- Wild Type: 5' CAAGATGACGATAAACCCAATTATTGAGCACGTAGGATACACAACC 3'

Y143F Primer 1: 5' CAAGATGACGATAAACCCAATTTTTGAGCACGTAGGATACACAACC 3'
Y143F Primer 1: 5' GGTTGTGTATCCTACGTGCTCAAAAATTGGGTTTATCGTCATCTTG 3'

- Wild Type: 5' CCAATTTATGAGCACGTAGGATACACAACCTTAAACAGAGAGCC 3'

Y148F Primer 1: 5' CCAATTTATGAGCACGTAGGATTCACAACCTTAAACAGAGAGCC 3'
Y148F Primer 1: 5' GGCTCTCTGTTTAAGGTTGTGAATCCTACGTGCTCATAAATTGG 3'

- Wild Type: 5' CTTAAACAGAGAGCCAGCATACAAAAAACATATGCCAGTC 3'

Y157F Primer 1: 5' CTTAAACAGAGAGCCAGCATTCAAAAAACATATGCCAGTC 3'
Y157F Primer 1: 5' GACTGGCATATGTTTTTTGAATGCTGGCTCTCTGTTTAAG 3'

The oligos were used in a PCR reaction as described above.

To eliminate template plasmid, 1 µl of *Dpn*1 restriction enzyme was added to the PCR mutagenesis product and this was incubated at 37 °C. After 2 hours this was transformed into competent bacteria and colonies were grown for mini-preparation as described in sections 2.3.3 and 2.3.4. DNA from the mini-preparations was sent for sequencing. A clone containing the mutation was re-transformed and DNA purified by maxi-preparation ready for transient transfections into mammalian cells.

2.4.6 Cloning of shRNA Sequences

ShRNA oligos were designed using Promega's shRNA design tool, to predict the most likely sequences to cause knock down, three sequences were chosen to test. A scrambled sequence was also chosen to act as a negative control. Oligos were from Sigma Genosys. Oligos include an upstream ACC overhang; a loop, TTCAAGAGA; a U6 termination sequence, TTTTT; and a downstream overhang and Pst1 partial site, C on the 5'-3' oligo and GACGT on the 3'-5' oligo. Oligos are as follows;

ShRNA oligos for α2-chimaerin;

Oligos for sequence 1:

5'ACCGACGATAAACCCAATTTATTTCAAGAGAATAAATTGGGTTTATCGTCTTTTTC 3'
5' TGCAGAAAAAGACGATAAACCCAATTTATTCTCTTGAAATAAATTGGGTTTATCGT 3'

Oligos for sequence 2:

5' ACCGGCCCTGACCCTGTTTGATTTCAAGAGAATCAAACAGGGTCAGGGCCTTTTTC 3'
5' TGCAGAAAAAGGCCCTGACCCTGTTTGATTCTCTTGAAATCAAACAGGGTCAGGGC 3'

Oligos for sequence 3:

5' ACCGGATACACAACCTTAAACATTCAAGAGATGTTTAAGGTTGTGTATCCTTTTTC 3'
5' TGCAGAAAAAGGATACACAACCTTAAACATCTCTTGAATGTTTAAGGTTGTGTATC 3'

Scrambled Sequence:

5' ACCGCTCTGACCAAATAAATTATTCAAGAGATAATTTATTTGGTCAGAGCTTTTTC 3'
5' TGCAGAAAAAGCTCTGACCAAATAAATTATCTCTTGAATAATTTATTTGGTCAGAG 3'

ShRNA oligos for Vps28;

Oligos for sequence 1:

5' ACCGGATATCACCCACAATAAATTCAAGAGATTTATTGTGGGTGATATCCTTTTTC 3'
5' TGCAGAAAAAGGATATCACCCACAATAAATCTCTTGAATTTATTGTGGGTGATATC 3'

Oligos for sequence 2:

5' ACCGAGCTGTATGAGGAAGTAATTCAAGAGATTACTTCCTCATACAGCTCTTTTTC 3'
5' TGCAGAAAAAGAGCTGTATGAGGAAGTAATCTCTTGAATTACTTCCTCATACAGCT 3'

Oligos for sequence 3:

5’ ACCGGCTCCTGGTCCAGTACAAATTCAAGAGATTTGTACTGGACCAGGAGCTTTTTC 3’
5’ TGCAGAAAAAGCTCCTGGTCCAGTACAAATCTCTTGAATTTGTACTGGACCAGGAG 3’

The system used was siSTRIKE U6 Hairpin Cloning System from Promega, which included linearised psiSTRIKE vectors containing the U6 promoter. This study used psiSTRIKE vectors encoding either neomycin resistance or hygromycin resistance. Oligonucleotides were diluted into nuclease free water to a concentration of 1 µg/µl. Annealing reactions were set up as follows;

- Oligonucleotide A 2 µl
- Oligonucleotide B 2 µl
- Oligo Annealing Buffer 46 µl (60 mM Tris-Hcl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$, 10 mM DTT.)

This mix was heated to 90°C for 3 minutes and then placed into a water bath at 37°C for 15 minutes. The annealed hairpin oligonucleotides were further diluted by adding 5 µl to 45 µl of nuclease free water. Ligations were set up as shown below;

- 2x rapid ligation buffer (vortexed before use) 5 µl
- psiSTRIKE vector (50 µg/ml) 1 µl
- Annealed oligonucleotides 1 µl
- Nuclease free water 2 µl
- T4 DNA ligase 1 µl

The ligation mixture was mixed by pipetting and incubated at room temperature for 2 hours. Transformation of competent bacteria was performed and colonies grown for mini-preparation as described in sections 2.3.3 and 2.3.4.

To screen for the insert, DNA was digested by Pst 1, a site incorporated into the vectors to allow screening for an insert. After Pst1 digestion, samples were checked by DNA electrophoresis and imaged via uv light and a Polaroid camera. Most samples contained the insert and therefore, one of each of the clones containing the shRNA insert sequence was picked to analyse. These were re-transformed, as described in sections 2.3.3 and 2.3.4, to ensure a clean colony and the DNA maxi-prepared ready for transient transfection into mammalian cells.

2.4.7 Cloning into Tap-Tag Vectors

The vectors pXJ40-8xHIS-precission-SBP and pBIOTIN-8HIS (pGEX-T4) were obtained from E. Manser, GSK-IMCB Singapore. These dual tagged His-Biotin tagged vectors enable proteins to be purified onto Nickel beads and subsequently purified via streptavidin to investigate endogenous partner proteins. Oligos were designed to amplify α2-chimaerin and the N-terminal region of α2-chimaerin including the SH2 domain (amino acids 1-137). Oligos included a stop codon TAG, a restriction site for cloning into the vector (*Xho1* and *Pst1* for the pXJ40 vector or *EcoR1* and *Sal1* for the pGEX vector) and an enzyme seat GACGTC. Oligos were designed as follows;

- pXJ40-8xHIS-precission-SBP

Forward Primer (including *Xho1* restriction site)

5’ GAC GTC CTC GAG ATG GCC CTG ACC CTG TTT G 3’

Reverse Primer (including *Pst1* restriction site)

For full length α2-chimaerin:

5'GA CGT CCT GCA GAT TTA AAA TAA AAT GTC TTC GTT TTT G 3'

For 1-137 α2-chimaerin:

5' GA CGT CCT GCA GGT CTA CAT CTT GGC AAT GTA TTC TGC TGC 3'

- pBIOTIN-8HIS (pGEX-T4)

Forward Primer (including *EcoR1* restriction site)

5' GAC GTC GAA TTC ATG GCC CTG ACC CTG TTT G 3'

Reverse Primer (including *Sal1* restriction site)

For full length α2-chimaerin:

5' GAC GTC GTC GAC AAA TAA AAT GTC TTC GTT TTT G 3'

For 1-137 α2-chimaerin:

5' GAC GTC GTC GAC CAT CTT GGC AAT GTA TTC 3'

The oligos were used in a PCR reaction for amplification as described previously. Once the PCR conditions (particularly the annealing temperature) had been established, a large scale PCR was set up and the product visualised via DNA gel electrophoresis and an ultra-violet light box. The product was cut out of the agarose gel and extracted and cleaned up using the QIAquick Gel Extraction Kit (from Qiagen) according to the manufacturer's instructions. Digests were set up as follows;

48 µl DNA

6 µl Enzyme

10 µl Appropriate Enzyme Buffer

1 µl BSA if required (or add 1 µl to the ddH_2O added)

35 µl ddH_2O

Both the vectors and the inserts required double digests, which were done sequentially with overnight incubations at 37 °C. Between each digest the enzymes were stopped from functioning and the DNA purified using the QIAquick Gel Extraction Kit. After the second digestion, the vectors were dephosphorylated. To 100 µl of vector digest, 13 µl of 10x buffer and 11 µl of 1:100 diluted alkaline phosphatase enzyme was added and incubated for 30 minutes at 37 °C. After this time, another 11 µl of 1:100 diluted alkaline phosphatase enzyme was added and again incubated for 30 minutes at 37 °C.

Vectors and insert sizes were checked via DNA electrophoresis before ligation. Ligations were set up as follows;

0.5 µl of Vector DNA

16.5 µl of Insert DNA (or ddH_2O for vector control)

2 µl of 10x T4 DNA Ligase Buffer

1 µl of Ligase Enzyme

The vector and insert DNA was denatured at 65 °C for 5 minutes, cooled on ice and the condensation centrifuged down. The buffer and enzyme were added, mixed on ice and incubated at 16 °C overnight. Ligation mixes were transformed as described in section 2.3.3 and *E. coli* colonies grown and the DNA purified by mini-preparation (Qiagen). The products were digested with the same enzymes used to clone the inserts, to check that the product contained the insert of the correct size. Once this was established and confirmed by DNA sequencing, the insert containing clone was retransformed and purified by maxi-preparation, ready for transient transfection into mammalian cells.

2.5 Protein Biochemistry Methods

2.5.1 Protein Expression Solutions

- **12% SDS Polyacrylamide Gel Electrophoresis (PAGE) gel-** Deionised water 10.2 ml, 30% Acrylamide stock solution 12 ml, 1.5 M TrisHCl pH 8.8 7.5 ml, 20 % SDS 150 µl, 10 % APS 150 µl, TEMED 20 µl.
- **4% SDS stack-** Deionised water 6.15 ml, 30% Acrylamide stock solution 1.34 ml, 0.5 M TrisHCl pH 6.8 2.5 ml, 20 % SDS 50 µl, 10 % APS 50 µl, TEMED 10 µl.
- **4x Protein Sample Buffer-** 200 mM TrisHCl pH 6.8, 8 % SDS, 40 % glycerol, 400 mM β-mercaptoethanol and 0.4 % Bromophenol blue made up in ddH_2O.
- **10x Tris-Glycine Running Buffer-** Tris Base 30 g, Glycine 144 g, SDS 10 g, make up to 1 L with ddH_2O.
- **10x Western Transfer Buffer-** 48 mM Tris Base, 39 mM Glycine, 1.3 mM SDS, and make up to 1 L with ddH_2O. To use, make 1x Transfer buffer as follows; 100 ml 10x Transfer buffer, 200 ml Methanol and 700 ml ddH_2O.
- **PBS-Tween-** 1 x PBS 1 L, Tween 0.1 %.
- **Blocking Buffer-** 5 % Marvel (powdered milk) in PBS-Tween.
- **Coomassie Stain-** Coomassie Brilliant Blue R250 0.5 g, Methanol:Water (1:1) 25 ml, Glacial acetic acid 50 ml, filter through Whatman filter paper.
- **Destain-** Methanol 450 ml, Glacial Acetic Acid 100 ml and Deionised Water 450 ml.

2.5.2 Analysis of Proteins by Polyacrylamide Gel Electrophoresis (PAGE)

Protein gels were made using a 12 % SDS separating gel, topped by a 4 % SDS stack. 10 µl of SeeBlue Pre-stained protein markers were loaded onto the gel to give an indication of protein size, once bands are identified. 4x Protein Sample Buffer was added to samples, which were heated at 95 °C for 10 minutes and 20 µl loaded onto the gel. Gels were run at 170 V for one hour.

2.5.3 Transferring Proteins to Polyvinylidene Difluoride (PVDF) Membranes

The protein was transferred from the polyacrylamide gel onto PVDF membrane using a Trans-blot semi-dry-transfer blotter (Biorad). The PVDF membrane was soaked in methanol and then as with the gels and blotting paper, soaked in 1x transfer buffer prior to being placed into the Trans-blot semi-dry-transfer blotter. Four pieces of 3 mm blotting paper were placed either side of the gel and membrane and any air bubbles rolled out. The Trans-blot semi-dry-transfer blotter was run at 22 V for 36minutes.

2.5.4 Coomassie Staining of Proteins

PVDF membranes were stained with coomassie stain for 5 minutes and placed in destain twice for ten minutes each. Proteins may be visualised in this way and background was reduced when PVDF membranes were subsequently used for immunodetection.

2.5.5 Immunodetection of Proteins Immobilised on PVDF Membranes

After de-staining, PVDF membranes were thoroughly washed with PBS. Membranes were placed in blocking buffer over night at 4 ˚C or for an hour at room temperature. Primary antibodies were added at the appropriate dilution (see antibody table) in 1 % Marvel in PBS-Tween and incubated over night at 4 ˚C or for two hours at room temperature. Membranes were washed five times with PBS-Tween and then incubated with secondary antibodies conjugated to Horse Radish Peroxidase (HRP) (for dilutions see antibody table, 2.1.8) also in 1 % marvel in PBS-Tween for one hour at room temperature. Membranes were washed five times with PBS-Tween before being incubated with ECL reagent for two minutes before being developed onto light sensitive film for detection.

For re-blotting, membranes were incubated in 1x Re-Blot Plus Strong Antibody Striping Solution (from Millipore) for 30 minutes at room temperature, thoroughly washed with PBS and then blocked as described above.

2.6 Cell Biology Methods

2.6.1 Cell Solutions

- **Growth Media-** DMEM, 10 % FCS and 1 % Antibiotic/antimycotic.
- **Freezing Solution-** 10 % DMSO and 90 % FCS.
- **Plating Media-** OptiMEM, 10 % FCS, 20 mM Glucose and 1 % Antibiotic/ antimycotic.
- **NB plus Media-** Neurobasal media, 1:50 dilution of B-27 supplement, 0.5 mM L-glutamine, 1 mM Sodium Pyruvate, 0.06 mg/ml Cysteine and 1 % Antibiotic/ antimycotic.
- **EB Lysis Buffer-** 20 mM TrisHCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 10 % glycerol and 1 % triton-X100 made up in ddH_2O.
- **N1E-115 Lysis Buffer-** 25 mM HEPES pH 7.5, 0.3 M NaCl, 1 mM MgCl, 1 mM EGTA, 20 mM p-glycerophosphate, 5 % glycerol and 1 % triton-X100 made up in ddH_2O.
- **Inhibitors-** 0.1 mM Sodium Vanadate, 10 mM Sodium Flouride, 5 mM DTT, 1x Protein inhibitor cocktail and 1 mM PMSF.

2.6.2 Recovery of Cells from Frozen Stocks

Vials of frozen cells were thawed from liquid nitrogen quickly in a 37 ˚C water bath. Cells were placed into pre-warmed media and centrifuged at 800 rpm for 5 minutes. The supernatant was removed and the cell pellet re-suspended in fresh media and plated onto Nunc coated dishes.

2.6.3 Freezing Down Cell Stocks

Cells were removed from the plate by pipetting or by trypsin treatment and centrifuged at 800 rpm for 5 minutes. Supernatant was removed and the pellet was resuspended in 1.5 ml of freezing solution and placed into a Cryovial. Cells were frozen down slowly to -20 ˚C and then to -80 °C over night before being placed into liquid nitrogen for storage.

2.6.4 Cell Maintenance

Three main cell types were used in this study, all cell types were maintained in incubators at 37 °C with 5 % CO_2.

COS-7 cells, derived from monkey kidney cells, were kept in media as listed above. To maintain the cells at the required density or to plate them for transfection, they were washed with PBS and treated with trypsin to detach them from the dish. The cells were centrifuged at 800 rpm for 5 minutes, the supernatant removed and the cells resuspended in media and distributed as required into dishes.

Mouse neuroblastoma N1E-115 cells were kept in media as above and maintained by never allowing them to become fully confluent. As loosely adherent cells, they do not require trypsin and can be pipetted off the dishes when required to plate them for transfection or for maintenance. N1E-115 cell lines permanently expressing receptors neuropilin-1, neuropilin-1/Plexin-A1 or Eph A4 were made during this study. These all had a neomycin selection gene encoded in their vectors to allow for selection of cells expressing the relevant receptor and were cultured in 4-800 µg/ml of the antibiotic G418.

The third cell type used were primary neuronal cells, hippocampal and cortical neurones isolated from E18 rat brains. These were isolated as described in section 2.6.11 and maintained by replacing half of their NB plus media every 2-3 days.

2.6.5 Preparation of Coverslips

Glass coverslips (24 x 24, thickness 1) were treated with 40 % HCl, 60 % ethanol for 10 minutes and washed thoroughly with water. Coverslips were placed out individually to dry on blotting paper, packaged into foil and baked overnight in an oven.

Coverslips were coated for use for 1 hour with 10 µg/ml laminin for N1E-115 neuroblastoma cells, or 30 µg/ml Poly-$_D$-Lysine (in ddH_2O) plus 2 µg/ml laminin (in PBS) for primary neurones. Coverslips were washed three times with water and allowed to dry before adding media and cells to them.

2.6.6 Transient Transfection of Cells

Cells were plated at around 70 % confluency. A DNA mix of 1 ml of DMEM, 2-5 µg of DNA and 15 µl of lipofectamine or lipofectamine 2000 (for Cos-7 and N1E-115 cells respectively) was made and left at room temperature for 45 minutes. Cells were serum starved in DMEM for this time and then the DNA mix was added for 4 hours. After this time, the media was replaced with the cell type's normal growth medium for 16 hours before harvesting.

2.6.7 Cell Treatments

- Phorbol 12-myristate 13-acetate (PMA)

PMA stock solution was made at 1 mM in DMSO and added to cells at 1 µM for 1 hour at 37 ˚C.

- Semaphorin 3A

Kindly given by Britta Eickholt (Eickholt, *et. al.,* 2002), Sema 3A-Fc enriched supernatent was added to cells at a 1:50 dilution for 10 minutes at 37 ˚C.

- Ephrin A1

Ephrin A1 (R&D Systems) (100 µg/ml stock in PBS) was clustered with goat-anti-human IgG at a 1:4.5 ratio for 1 hour at room temperature, before being added to cells at 5 µg/ml for 10 minutes at 37 ˚C.

- PP2/PP3

The Src family kinase inhibitor PP2 (Sigma) and its inactive analogue PP3 were added to cells at 20 µM for 1 hour at 37 ˚C.

- Pervanadate

Pervandate is a phosphatase inhibitor. A pervanadate stock was made; 593 µl PBS, 60 µl 200 mM Na_3VO_4, 1 µl 30 % w/w H_2O_2, and left at room temperature for 5 minutes. A 20 mg/ml catalase stock was made; with an equal volume of ddH_2O and 0.1 M PPB and 0.01 g of catalase added per 500 µl. 0.6 g of the catalase stock was added to the pervanadate stock and left at room temperature for 5 minutes. This was added to cells at a 1:200 dilution for 20 minutes at 37 ˚C.

2.6.8 Affinity Purification of Proteins (by pull down assay)

Dishes containing COS-7 cells expressing tagged proteins were placed on ice, media was removed and the cells washed with PBS. 500 µl of EB lysis buffer with inhibitors freshly added was added to the dish and incubated for 5 minutes on ice. Cells were scraped off the dish and pipetted into eppendorf tubes. The samples were sonicated in a sonicator ultrasonic processor (Heat Systems) at level 6 for 5 minutes and incubated on ice for 30 minutes. Samples were pelleted in a 4 ˚C centrifuge at 14,000 g for 20 minutes.

Dishes containing N1E-115 cells expressing tagged proteins were placed on ice, media was removed and 500 µl of N1E-115 lysis buffer with inhibitors freshly added was added to the dish. Cells were scraped off the dish and pipetted into eppendorf tubes. The samples were incubated on ice for 15 minutes, and then pelleted at 4 ˚C at 14,000 g for 15 minutes.

Following centrifugation, for both COS-7 and N1E-115 neuroblastoma cells, a 100 µl supernatant sample was collected, 4x protein sample buffer was added and these were frozen at -20 ˚C. The rest of the supernatant was transferred to a new tube and incubated with 30 µl of the appropriate affinity beads (FLAG or HA antibody) for 3 hours at 4 ˚C on a rotary shaker. Beads were washed 5 times in EB lysis buffer (COS-7 cells) or N1E-115 lysis buffer (N1E-115 cells) with the inhibitors freshly added and after the final wash beads were pelleted, all supernatant removed and 4x protein sample buffer added. Samples were frozen for later analysis by SDS-PAGE.

2.6.9 Immunoprecipitations

To immunoprecipitate α2-chimaerin, cells were lysed as described in protein pull downs. 1 µg of rabbit IgG and 20 µl of rabbit True-blot or protein A/G beads were added to pre-clear the supernatant for 30 minutes at 4 °C. Beads were pelleted in a 4 °C centrifuge and the supernatant moved to a new tube. α2-Chimaerin antibody was added to the supernatant for an hour at 4 °C. A fresh 20 µl of beads was added and the tube rotated at 4 °C for ~ 3 hours. Beads were washed 4x with PBS and after the last wash, the supernatant removed and sample buffer added. Samples were then frozen for later analysis by SDS-PAGE.

2.6.10 His-Biotin-Tandem Affinity Purification from Brain Extracts

To pull down α2-chimaerin using the His-Biotin vector tags, BL-21 cells were transformed with α2-chimaerin His-Biotin onto an agar plate and incubated at 37 °C overnight. Around 30 of the colonies were inoculated into 400 ml of LB media supplemented with ampicillin and grown at 37 °C until an absorbance of OD_{600} = 1 was achieved. IPTG 0.25 mg/ml and D-Biotin 1 µg/ml was added for a further 3 h of agitation at room temperature. Cells were centrifuged for 10 minutes at 6000 rpm at 4 °C. The pellet was stored overnight at -80 °C.

The following day, the pellet was thawed on ice and subsequently resuspended in 40 ml of cold bacteria cell lysis buffer (50 mM Tris (pH 8.0), 0.5 % Triton X-100, 0.5 mM $MgCl_2$, 10 mM Imidazole and PBS. Protease inhibitors were added before use). For 5 minutes. Cells were sonicated twice for a total of 1 minute each, with 20 seconds of

sonication and 20 seconds pause. Cells were centrifuged at 40,000 rpm, 4 °C for 30 minutes.

The supernatant was loaded onto a nickel column (GE Healthcare), washed twice with a column volume of cold washing buffer (PBS, 25 mM Tris (pH 7.5) and 0.1 % Triton X-100) and 20 mM Imidazole. The His-tagged fusion protein was eluted with 50 mM Tris.Cl (pH 8.5), 100 mM NaCl and 250 mM Imidazole.

The eluted protein was passed through a streptavidin binding column (from IBA) and washed three times with cold washing buffer and 0.1 µg/ml of Biotin to block non-specific sites. Previously prepared synaptosomal membrane fractions of rat brain extract was passed through the column twice and subsequently washed three times with cold washing buffer. The interactions were eluted with desthiobiotin, then 1 % SDS sample buffer at 37 °C and subsequently 2 % SDS sample buffer at 37 °C. Samples were analysed via SDS-PAGE.

2.6.11 Neuronal Preparation

The hippocampus or cortices were dissected from E18 rat brains, all tissue was kept in ice cold Hanks buffered saline solution (HBSS). Once dissected, the tissue was suspended in 2 ml of HBSS, 0.2 µg/ml of DNase 1 and 1x trypsin and incubated at 37 ˚C for 15 minutes. This mix was removed and the tissue washed three times with 1 ml of plating media. The last wash was removed and a fresh 1 ml of plating media added. Using two glass Pasteur pipettes which had their holes reduced in diameter (one more than the other) by holding them in a flame, the tissue was dissociated by passing them first through the larger and then the smaller pipettes until a homogeneous cell suspension without tissue

particles was obtained. 9 ml of plating media was added and the cells counted and centrifuged for a few minutes at 2-300 rpm. Cells were resuspended in plating media and plated at around 200,000 cells per coverslip, media was replaced with NB plus media after 2-3 hours, or if cells were to be transfected by electroporation, cells were treated as described below (Section 2.6.12).

2.6.12 Electroporation of Neuronal Cells

After centrifugation, the cell pellet was resuspended in 100 µl of rat neuron nucleofector solution (Amaxa) per transfection reaction, with 1-2 million cells per reaction. 2 µg of DNA was put into the electroporation cuvettes and 100 µl of the resuspended cells added to it. Cuvettes were put into the Nucleofector II and run on programme 0-003 a programme designed for primary rat hippocampal neurones. 100 µl of plating media was added to the cuvettes to dilute the Nucleofector solution and the whole mix was removed from the cuvette with a sterile plastic pipette and the cells plated onto coverslips in plating media. After 2-3 hours the plating media was changed to NB plus media.

2.6.13 Cell Immunostaining

Cells were fixed in 4 % paraformaldehyde (PFA) in PBS for 10 minutes. This was washed off with two 10 minute PBS washes. Cells were permeabilised in 0.2 % triton in PBS for 7.5 minutes and blocked in 3 % BSA in PBS for 15 minutes. Primary antibodies were added at the appropriate dilution (see antibody table, section 2.1.8) in 1 % BSA in PBS. The coverslips were covered with parafilm, to prevent drying out, and dishes wrapped in foil before being placed in a 37 ˚C incubator for a 2 hour incubation. Cells

were washed 3 times for 10 minutes each in PBS. Secondary antibodies were added at the appropriate dilution (see antibody table, section 2.1.8) and Phalloidin was added at 1:100 in 1 % BSA in PBS. Again the coverslips were covered with parafilm and the dishes wrapped in foil for a one hour incubation at 37 ˚C. Cells were washed three times for 10 minutes each in PBS. The coverslips were dried and then mounted onto slides and analysed by microscopy.

2.6.14 Microscopy

Cells were visualized using an Zeiss Axioplan or Zeiss LSM 510 confocal microscope. Using FITC (488 nm), TRITC (568 nm) and Cy5 (633 nm) excitation channels.

Chapter 3.0:

Results I:

Mechanisms of α2-Chimaerin Activation and Tyrosine Phosphorylation by Fyn

3.1 Mechanisms of α2-Chimaerin Activation

Axons and dendrites of developing neurones follow attractive and repulsive guidance cues to establish neuronal networks. Guidance cues include the ephrin and semaphorin families of repulsive signalling molecules, which cause axonal growth cones to collapse, enabling developing axons and dendrites to avoid inappropriate environments. Receptors for guidance signals activate the Rho GTPase family of proteins which regulate cytoskeleton remodelling. The Rho GTPase Rac1 is essential in the sema 3A growth gone collapse pathway (Jin and Strittmatter, 1997). Rac1 activation is also required in driving ephrin A1/EphA receptor and sema 3A mediated endocytosis of the plasma membrane (Jurney *et. al*., 2002). The Rac1 GAP, α2-chimaerin was previously found to be involved in the sema 3A collapse pathway, associating with other essential components (Brown *et. al*., 2004) and during the course of this study, α2-chimaerin was subsequently shown to be important in ephrin/EphA4 collapse (Wegmeyer *et. al.*, Beg *et. al.*, Iwasato *et. al.* and Shi *et. al.*, 2007). The SH2 and GAP domains of α2-chimaerin are both essential in the sema 3A collapse process (Brown *et. al.*, 2004), as well as in ephrin /EphA4 signalling (Iwasato *et. al.*, 2007; Shi *et. al.*, 2007).

DAG binding to the C1 domain of chimaerin is known to cause chimaerin activation (Caloca *et. al.*, 1997). However, it is unclear how α2-chimaerin is activated by receptor signalling in collapse pathways, and it is possible that tyrosine phosphorylation of α2-chimaerin may be involved. The interactions of the SH2 domain in α2-chimaerin in these signalling pathways, possibly involving tyrosine phosphorylated target proteins, are unknown. This study aimed to investigate these interactions and to determine whether α2-chimaerin is itself tyrosine phosphorylated. Additionally, this study aimed to assess

whether phosphorylation could be a mechanism of activation for α2-chimaerin in sema 3A and ephrin A1 collapse pathways or whether C1 domain interactions were required.

3.2 DAG/Phorbol Ester Activation of α2-Chimaerin in COS-7 Cells

Chimaerin GAP activity is tightly regulated. The phorbol ester, PMA, binding to the C1 domain is a known mechanism of activation (Ahmed *et. al.*, 1993). A single point mutation in the C1 domain of β2-chimaerin blocks phorbol ester/DAG binding and therefore, β2-chimaerin activation by phorbol ester (Caloca *et. al.*, 1999).

The importance of DAG activation in physiological pathways is not fully established. To aid investigation of DAG-regulated cellular functions of α2-chimaerin, the equivalent cysteine in α2-chimaerin, C239, was mutated to alanine and verified by sequencing, as described in Methods (Section 2.4.5). To test whether this mutation was sufficient to block α2-chimaerin activation by the phorbol ester PMA, COS-7 cells were transfected with wild type α2-chimaerin or the C239A mutant and either untreated or treated with PMA.

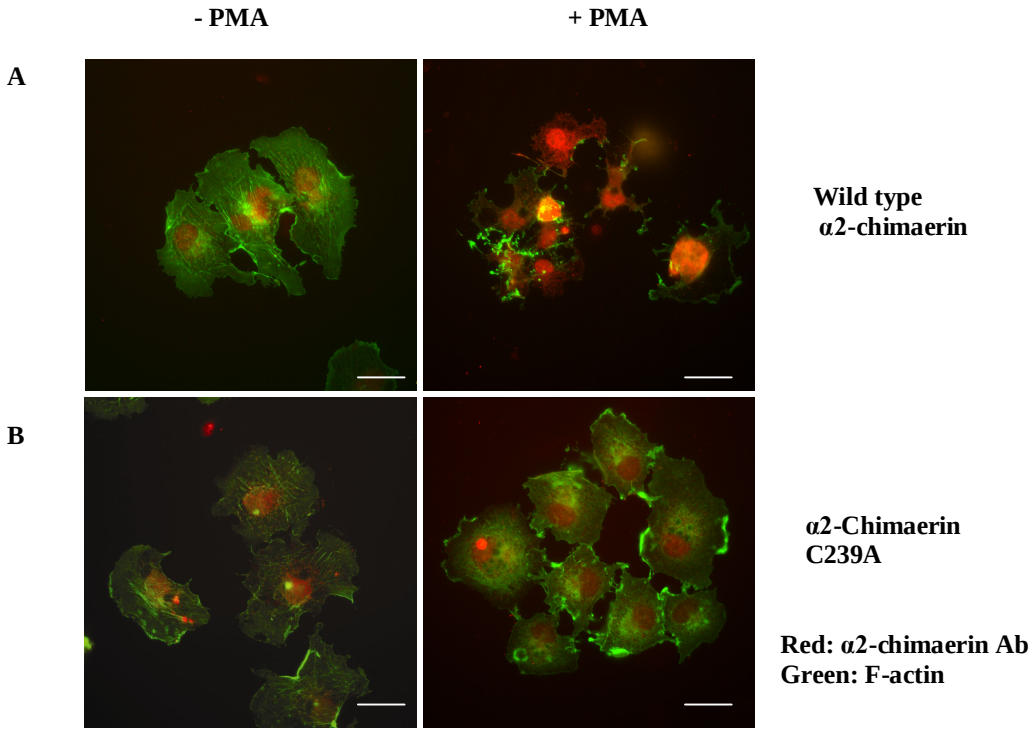


Figure 3.1: C239A mutation of α2-chimaerin is sufficient to prevent activation by PMA COS-7 cells were transfected with α2-chimaerin or C1 domain mutant α2-chimaerin C239A. Cells were untreated, or treated with 1 µM PMA, fixed with 4 % PFA and stained for α2-chimaerin with rabbit α2-chimaerin antibody and TRITC-secondary antibody (red) and F-actin stained with phalloidin-FITC (green). The scale bar represents 10 µm.

Cells that were transfected with α2-chimaerin and treated with PMA show a collapsed state distinctly lacking F-actin, compared with PMA untreated cells (Figure 3.1 A). Whereas, the cells transfected with chimaerin C239A and treated with PMA do not collapse (B), but appear ruffled indicating that this single point mutation is sufficient to prevent PMA from activating α2-chimaerin (Figure 3.1 B). PMA treatment of COS-7 cells induces cell ruffling, a Rac1 response (Ridley *et. al.*, 1992; Kozma *et. al.*, 1996). The Rac1 ruffling response was prevented by exogenous α2-chimaerin activated by PMA down regulating Rac1 (Figure 3.1 A). Membrane ruffling in cells expressing α2-

chimaerin C239A (Figure 3.1 B) is consistent with this mutation preventing RacGAP activation by PMA, allowing its use in further experiments.

Figure 3.1 also shows α2-chimaerin localising to the nucleus (red staining). As this localisation was observed with this antibody on occasion throughout this study and by other users, it is possible that α2-chimaerin may have some transient movement into the nucleus, or that a component of this α2-chimaerin antibody (raised against recombinant protein synthesized in *E. coli* (Hall *et. al.,* 2001)) cross-reacts with a component in the cell nucleus.

3.3 Tyrosine Phosphorylation of α2-Chimaerin

Previous results in the laboratory have shown that the N-terminal region of purified recombinant α2-chimaerin can be tyrosine phosphorylated *in vitro* by Src. Because of the auto-inhibited structure of β2-chimaerin, conserved in α2-chimaerin, the N-terminal and SH2 domain regulate the GAP activity (Canagarajah *et. al,* 2004) and it is possible that tyrosine phosphorylation of the N-terminal region of chimaerin may be involved in its regulation. To determine whether tyrosine phosphorylation of α2-chimaerin occurs *in vivo*, the two known growth cone collapse signalling pathways in which α2-chimaerin is essential, sema 3A and ephrin A1/EphA4, were investigated. Initial experiments were undertaken with sema 3A because they pre-dated the discovery of chimaerin association with EphA4 signalling.

3.3.1 Tyrosine Phosphorylation of α2-Chimaerin in Response to Semaphorin 3A

To investigate α2-chimaerin tyrosine phosphorylation in sema 3A signalling, the possibility of a permissive cell system was explored. N1E-115 neuroblastoma cells express α2-chimaerin and are one of the best characterised neuronal cell lines in relation to morphology and Rho GTPase function (Zhang *et. al.*, 1996; Kozma *et. al.*, 1996). N1E-115 neuroblastoma cells are capable of responding to sema 3A (Van Horck *et. al.*, 2002).

Figure 3.2 demonstrates a series of preliminary experiments investigating tyrosine phosphorylation of α2-chimaerin in N1E-115 neuroblastoma cells. N1E-115 cells were treated with a 1:50 dilution of sema 3A-Fc enriched cell media supernatent (Eickholt *et. al.*, 2002) over a time course of 0-30 minutes, lysed and total lysates analysed by SDS-PAGE and immunodetection with a phosphotyrosine antibody (PY20) (Figure 3.2A). There was no overall stimulation of protein phosphorylation. To determine whether tyrosine phosphorylation of α2-chimaerin in response to sema 3A could be detected, N1E-115 neuroblastoma cells were transfected with full length FLAG tagged α2-chimaerin, Na_3VO_4 (a tyrosine phosphatase inhibitor) was added to the cells to enhance phosphorylation with a sema 3A time course of 0-60 minutes (Figure 3.2B).

Src family tyrosine kinases are widely involved in many signalling pathways, and Fyn is a known component of sema 3A pathways (Sasaki *et. al.*, 2002; Morita *et. al.*, 2006). To test whether increased tyrosine phosphorylation of α2-chimaerin was due to Src family kinases, N1E-115 neuroblastoma cells were transfected with FLAG-tagged α2-chimaerin and treated with Na_3VO_4 and sema 3A, either in the absence or presence of the Src family kinase inhibitor PP2 (Figure 3.2C).

In figures 3.2B and C, α2-chimaerin was affinity purified onto FLAG antibody beads and analysed by SDS-PAGE and western immunoblotting with a phosphotyrosine antibody and subsequently re-blotted with α2-chimaerin antibody.

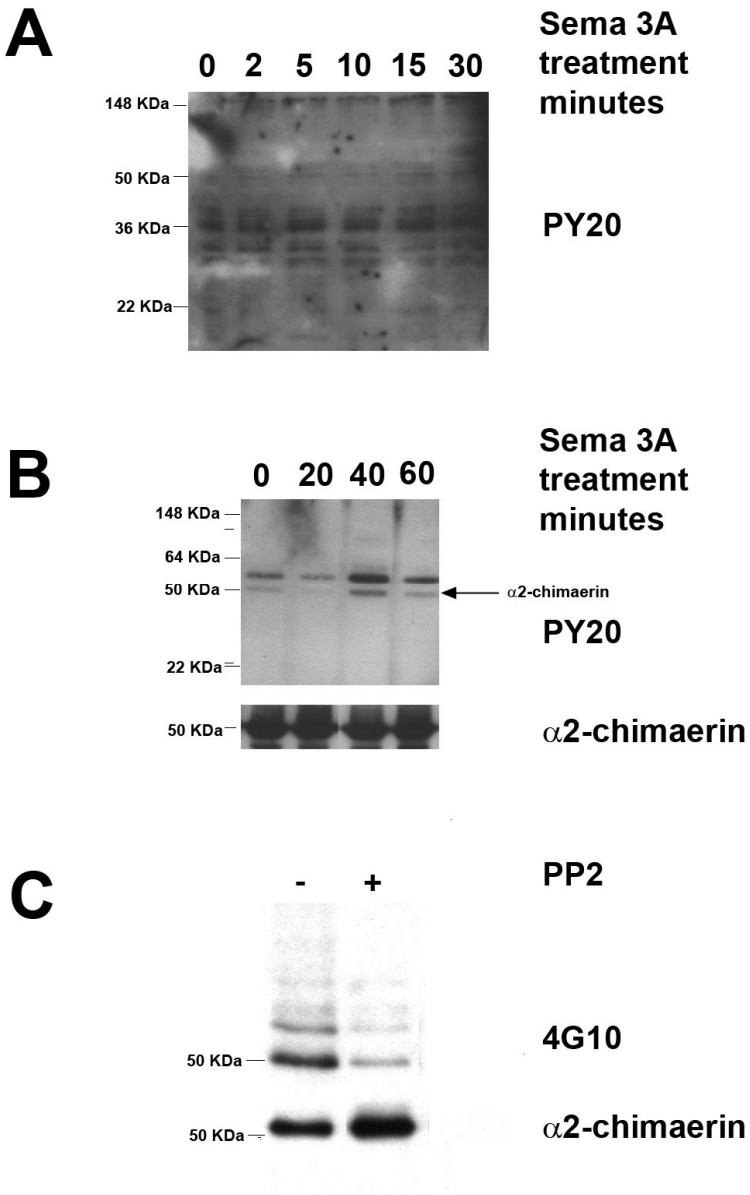


Figure 3.2: Series of preliminary experiments investigating tyrosine phosphorylation of α2-chimaerin in N1E-115 neuroblastoma cells
(A) N1E-115 neuroblastoma cells were treated with sema 3A at a 1:50 dilution at times indicated, cells were lysed, sample buffer added to the total lysate before analysis on an SDS-PAGE gel and proteins transferred onto PVDF membrane were immunodetected with the phosphotyrosine antibody PY20.

(B) N1E-115 neuroblastoma cells were transfected with FLAG tagged α2-chimaerin and treated with Na_3VO_4 and with sema 3A at the times indicated. Cells were lysed and incubated with FLAG beads, to pull down α2-chimaerin. Samples were analysed by SDS-PAGE and western blotted with phosphotyrosine antibody PY20 and subsequently re-blotted with α2-chimaerin antibody.
(C) N1E-115 neuroblastoma cells were transfected with FLAG tagged α2-chimaerin, treated with Na_3VO_4 for 20 min, and treated with sema 3A for 40 min, either in the absence or presence of PP2. Cells were lysed and α2-chimaerin affinity purified using FLAG beads. Samples were analysed by SDS-PAGE and western blotted with phosphotyrosine antibody 4G10 and subsequently re-blotted with α2-chimaerin antibody.

Although in N1E-115 neuroblastoma cell total lysate there was no net increase in tyrosine phosphorylation of total lysate proteins (Figure 3.2A), phosphorylation of α2-chimaerin was detected, highest after 40 minutes of sema 3A treatment (Figure 3.2B). The phosphotyrosine level was reduced in the presence of PP2, suggesting that Src family tyrosine kinases may be at least partially involved in this tyrosine phosphorylation of α2-chimaerin (Figure 3.2C), although figure 3.2C is lacking a specific control for PP2 inhibition. Later experiments showed that the upper band in Figures 3.2B and C is likely to be Fyn.

Overall, this preliminary data shows that it is possible to detect phosphorylated α2-chimaerin when affinity purified using FLAG beads and that phosphorylation is sensitive to PP2. However, sema 3A stimulation produced only slight tyrosine phosphorylation of α2-chimaerin, possibly due to low level endogenous receptors. An N1E-115 neuroblastoma cell line over-expressing the neuropilin 1/plexin A1 receptor complex was produced, which did show tyrosine phosphorylation following sema 3A stimulation after a short time course, typical of receptor signalling. Vanadate was also not required, however, stimulation induced apoptosis and therefore work with this cell line was discontinued.

3.3.2 α2-Chimaerin is Phosphorylated by Fyn

To test whether Fyn can directly tyrosine phosphorylate α2-chimaerin, Fyn or kinase dead Fyn (K299M) were co-expressed with FLAG-tagged α2-chimaerin in N1E-115 neuroblastoma cells, and cells were either untreated or treated with PMA. cDNA in pCMV5 encoding Fyn and kinase-inactive Fyn K299M were kindly provided by M. Resh (Sloane-Kettering Cancer Centre, New York).

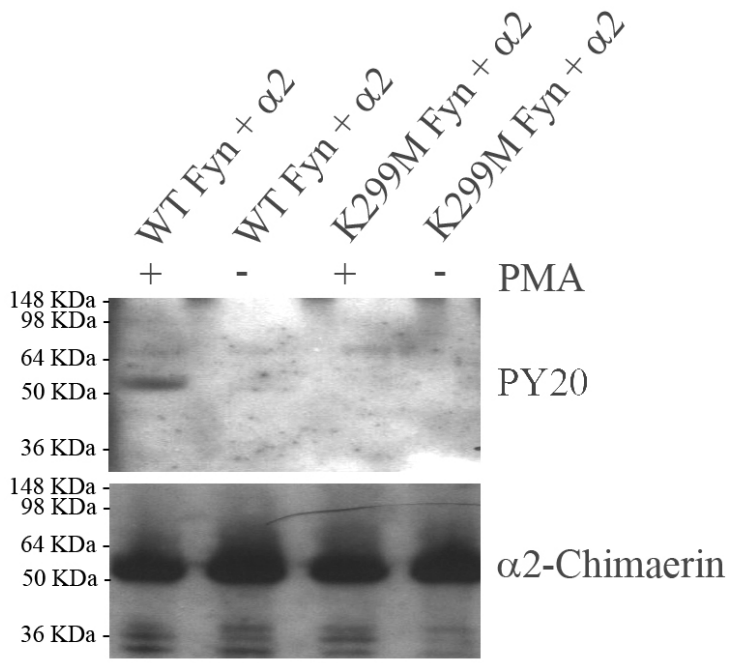


Figure 3.3: Wild type Fyn can phosphorylate α2-chimaerin in the presence of PMA N1E-115 neuroblastoma cells were transfected with FLAG-α2-chimaerin and either Fyn or kinase dead Fyn K299M. Cells were untreated or treated with 1μM PMA for 1 h. Cells were lysed and α2-chimaerin affinity purified onto FLAG antibody beads. Samples were analysed by SDS-PAGE and immunodetected with phosphotyrosine antibody PY20 and α2-chimaerin antibody.

Results showed that Fyn phosphorylated α2-chimaerin, only after treatment with PMA (Figure 3.3). There was no phosphorylation with kinase inactive Fyn or in the absence of

PMA. This implies that α2-chimaerin must be in an open conformation (with PMA treatment) to become tyrosine phosphorylated. Phosphorylation may therefore be an indication of activation.

To test whether sema 3A treatment can activate α2-chimaerin enough to be phosphorylated by Fyn, N1E-115 neuroblastoma cells were co-transfected with wild type Fyn and α2-chimaerin. Cells were untreated, PMA-treated or sema 3A-treated. α2-Chimaerin was affinity purified onto FLAG antibody beads and samples analysed via SDS-PAGE and western blotting using the anti-phosphotyrosine antibody 4G10.

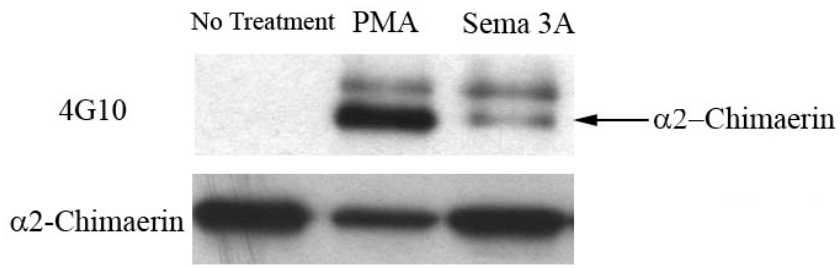


Figure 3.4: Fyn phosphorylates α2-chimaerin after cell treatment with chimaerin activators PMA and sema 3A N1E-115 neuroblastoma cells were transfected with FLAG tagged α2-chimaerin and Fyn. Cells were untreated, treated with PMA or treated with sema 3A. Cells were lysed and α2-chimaerin affinity purified onto FLAG antibody beads. Samples were analysed by SDS-PAGE and western blotted with 4G10 phosphotyrosine and α2-chimaerin antibodies.

In untreated cells no α2-chimaerin phosphorylation was observed, however, after treatment either with PMA or sema 3A, α2-chimaerin was phosphorylated by Fyn, although to a much lesser extent with sema 3A than with PMA (Figure 3.4). α2-Chimaerin was affinity purified onto FLAG-antibody beads, the upper tyrosine

phosphorylated band was always detected in these experiments along with α2-chimaerin tyrosine phosphorylation and has the same mobility as Fyn, suggesting Fyn may bind α2-chimaerin.

To investigate whether Fyn and α2-chimaerin interact when co-expressed in N1E-115 neuroblastoma cells, α2-chimaerin was affinity purified onto FLAG antibody beads and proteins that were pulled down with α2-chimaerin were probed with anti-Fyn antibody. Samples were either untreated or treated with PMA (Figure 3.5).

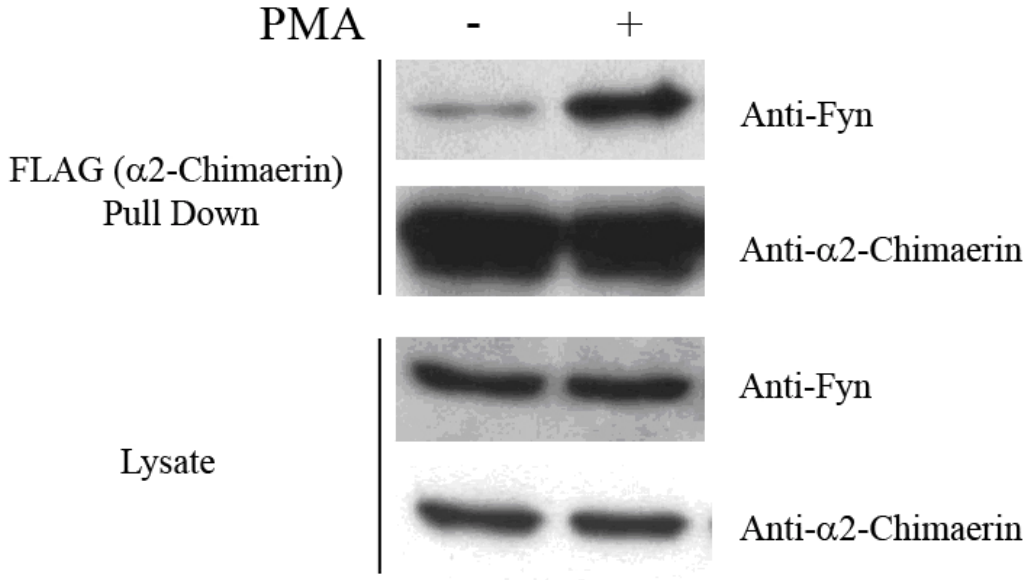


Figure 3.5: Fyn interacts with α2-chimaerin N1E-115 neuroblastoma cells were transfected with FLAG tagged α2-chimaerin and wild type Fyn. Cells were treated with sema 3A and either untreated or treated with PMA. α2-Chimaerin was affinity purified onto FLAG-antibody beads. Samples were analysed by SDS-PAGE and immunodetected with Fyn and (re-blotted with) α2-chimaerin antibodies.

Results showed that Fyn interacted with α2-chimaerin and this was enhanced by PMA treatment (Figure 3.5).

The region of α2-chimaerin that interacted with Fyn was also investigated, using FLAG-tagged fragments of α2-chimaerin. The fragments of α2-chimaerin that were available for use are shown in figure 3.6.

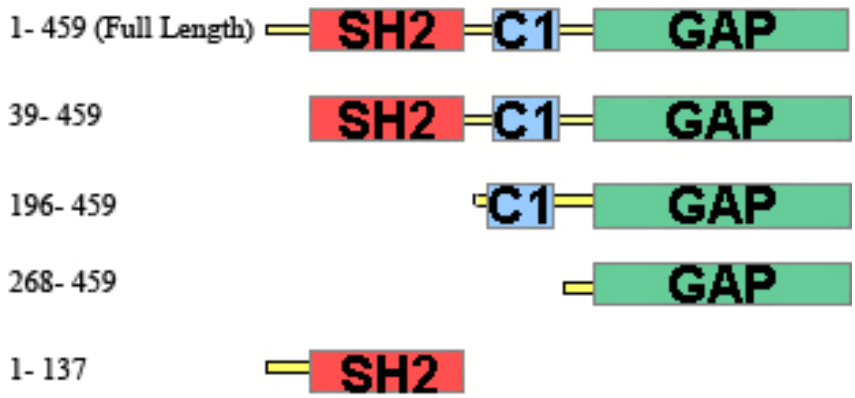


Figure 3.6: Fragments of α2-chimaerin A representation of the FLAG-tagged fragments of α2-chimaerin that were used in pull down experiments.

N1E-115 neuroblastoma cells were co-transfected with Fyn and FLAG-tagged α2-chimaerin or α2-chimaerin fragments. Cells were subsequently treated with sema 3A and subjected to FLAG-pull down assays (Figure 3.7).

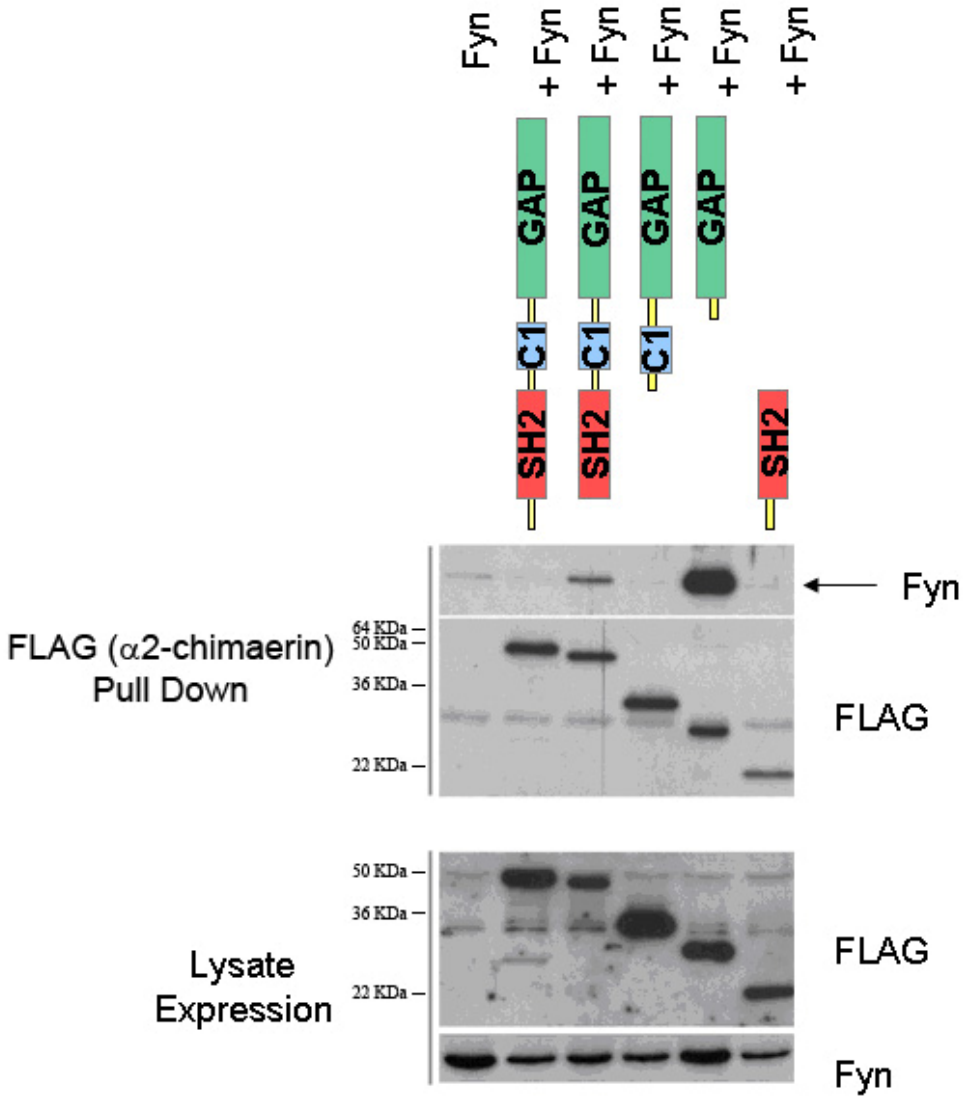


Figure 3.7: Fyn interacts with α2-chimaerin GAP domain N1E-115 neuroblastoma cells were transfected with FLAG tagged, α2-chimaerin fragments and Fyn. Cells were lysed and incubated with FLAG antibody beads to pull down α2-chimaerin. Samples were analysed by SDS-PAGE and immunodetected with Fyn and FLAG antibodies. This experiment was repeated, producing the same results on three occasions.

Fyn associated strongly with the α2-chimaerin GAP domain, amino acid residues 268-459. Fyn also pulled down with α2-chimaerin 39-459 (Figure 3.7). The N-terminal deletion (1-39) of α2-chimaerin is likely to release the auto-inhibited structure into an open conformation and may therefore make the GAP domain more available for binding

than wild type α2-chimaerin. Surprisingly, Fyn failed to bind to the fragment containing amino acids 196-459, comprising the C1 and GAP domains, perhaps the conformation of this fragment prevents Fyn from binding in some way. A small amount of background can be seen on the FLAG antibody beads alone.

3.3.3 Tyrosine Phosphorylation of α2-Chimaerin in the EphA4 Pathway

During the course of this study, collaboration was set up with Nancy Y. Ip in Hong Kong (Shi *et. al.,* 2007). Several studies, including this one, described α2-chimaerin in receptor tyrosine kinase EphA4 forward signalling (Wegmeyer *et. al.,* 2007; Beg *et. al.,* 2007; Iwasato *et. al.,* 2007; Shi *et. al.,* 2007). Ephrin/EphA4 signalling, like sema 3A signalling, is a growth cone collapse pathway important in neuronal development and axonal guidance. To investigate α2-chimaerin and Fyn in this pathway we obtained cDNA clones of the EphA4 receptor and a kinase dead mutant of the EphA4 receptor from N. Y. Ip (See Materials and Methods, Chapter 2.0). To be effective, the soluble ligand ephrin A1 conjugated to the Fc portion of IgG (R & D Systems) required treatment with goat-anti-human IgG (Sigma-Aldrich), for an hour at room temperature, to promote clustering; this allowed the study of EphA4 forward signalling.

To investigate whether α2-chimaerin is tyrosine phosphorylated following treatment with clustered ephrin A1, N1E-115 neuroblastoma cells were co-transfected with either wild type or kinase dead EphA4 and either wild type α2-chimaerin or α2-chimaerin C239A. Src family kinases are also a component of the EphA4 pathway (Ellis *et. al.,* 1996; Shi *et. al.,* 2007), and earlier results showed Fyn phosphorylated α2-chimaerin only after

treatment of α2-chimaerin activators PMA and sema 3A (Figure 3.4). Therefore, the effect of over-expressed Fyn was also investigated in ephrin signalling. To induce EphA4 signalling, cells were treated with pre-clustered ephrin A1 for 10 minutes. FLAG-α2-chimaerin was affinity purified onto FLAG antibody beads prior to western blot analysis with 4G10 phosphotyrosine antibody.

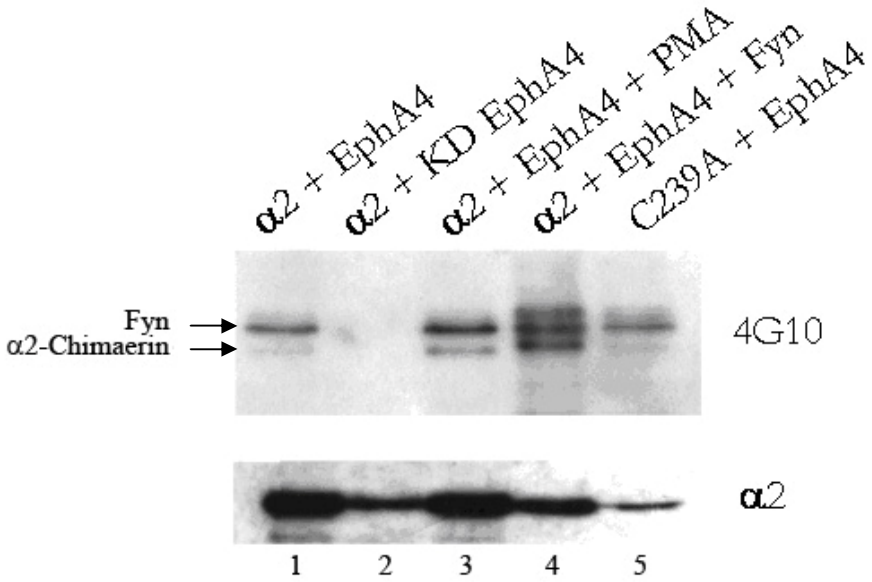


Figure 3.8 α2-Chimaerin phosphorylation occurs downstream of ephrin A1/EphA4 in N1E-115 cells and is enhanced by co-expression of Fyn N1E-115 neuroblastoma cells were co-transfected with Flag-α2-chimaerin or FLAG-α2-chimaerin C239A and either wild type EphA4, Kinase Dead (KD) EphA4 and Fyn as indicated. Cells were treated for 10 minutes with pre-clustered ephrin A1 before lysis and incubation with FLAG antibody beads to pull down FLAG-tagged α2-chimaerin. Samples were immunodetected with phosphotyrosine 4G10 and subsequently re-blotted with α2-chimaerin antibody.

Slight tyrosine phosphorylation of α2-chimaerin occurred in cells co-expressing EphA4 receptor and α2-chimaerin, following treatment with pre-clustered ephrin A1. Tyrosine phosphorylation of α2-chimaerin was enhanced by treatment with PMA or by the addition of co-transfected Fyn, where additional bands were co-purified. No tyrosine

phosphorylation was observed in cells co-transfected with kinase dead EphA4 and α2-chimaerin (Figure 3.8). The putative Fyn band was more strongly phosphorylated than chimaerin in all samples, with the exception of the kinase-dead EphA4. The C1 mutant chimaerin C239A, although expressed at a lower level (lane 5), was also capable of being phosphorylated, indicating that a functional DAG binding domain is not essential.

3.3.4 Tyrosine Mutations of α2-Chimaerin

To investigate sites in α2-chimaerin that are candidates for Fyn tyrosine phosphorylation, tyrosine residues were mutated. There are eleven tyrosine residues in the N-terminal fragment of α2-chimaerin which was previously Src-phosphorylated *in vitro* in our laboratory, the most likely tyrosine phosphorylation sites were determined using online phosphorylation prediction site: http://www.cbs.dtu.dk/services/NetPhos/. Four tyrosine residues in the SH2 domain and flanking region, which are conserved between species, scored highly as likely sites for tyrosine phosphorylation and therefore they were chosen for further study. α2-chimaerin Y70, Y143, Y148 and Y157 were separately mutated to phenylalanine to prevent phosphorylation. In addition, Y303 in the GAP domain adjacent to the essential arginine R304 was also mutated to phenylalanine. All mutations were made by site directed mutagenesis and confirmed by sequencing (see Methods). Figure 3.9 demonstrates the locations of mutated tyrosines in α2-chimaerin (red) and of mutations inactivating each of the domains (blue). The C1 domain inactivating mutation C239A was described earlier (Figure 3.1). Mutations inactivating the other two domains of α2-chimaerin were already available in our laboratory (Hall *et. al.*, 2001); α2-chimaerin R73L in the SH2 domain, preventing tyrosine binding and α2-chimaerin

R304G in the GAP domain inhibiting GAP activity. The domain inactivating mutations were also used in this part of the study investigating activation/ tyrosine phosphorylation of α2-chimaerin.

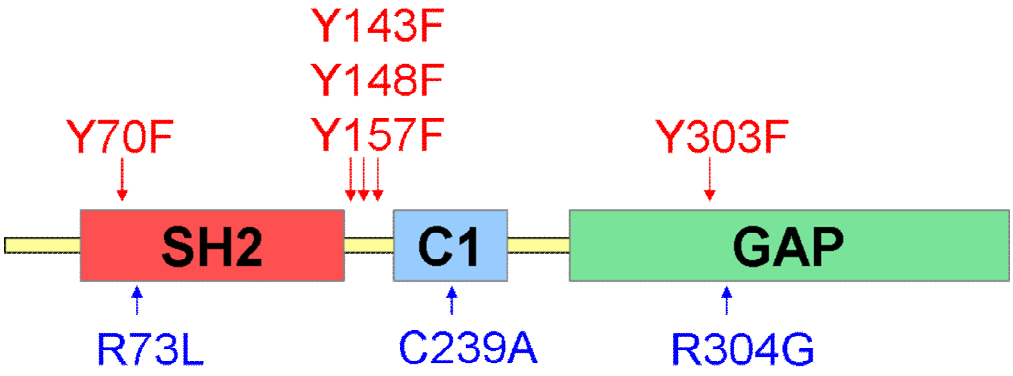


Figure 3.9: Mutations of α2-chimaerin Diagram showing the position of tyrosine mutations of α2-chimaerin (red text) and domain inactivating mutations of α2-chimaerin (blue text), used in this part of the study to investigate tyrosine phosphorylation of α2-chimaerin.

Two different phosphotyrosine antibodies were tested, PY20 and 4G10 (See Antibody table in Materials) (Figure 3.10). N1E-115 neuroblastoma cells were co-transfected with FLAG-tagged α2-chimaerin and either K299M (kinase dead) or wild type Fyn and treated with PMA for 1 hour. α2-Chimaerin was affinity-purified onto FLAG antibody beads and samples analysed by western blot using anti-phosphotyrosine antibodies PY20 or 4G10 as indicated.

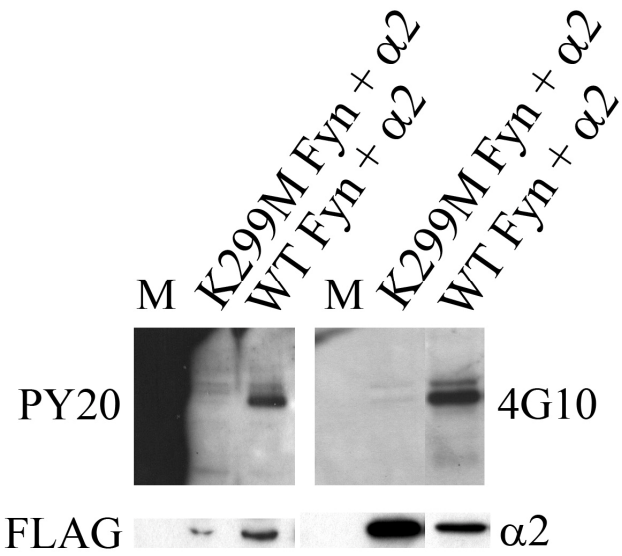


Figure 3.10: Comparison of phosphotyrosine antibodies N1E-115 neuroblastoma cells were transfected with FLAG tagged, full length α2-chimaerin and either K299M (kinase dead) or wild type Fyn. Cells were treated with PMA and α2-chimaerin affinity purified onto FLAG-antibody beads. Samples were analysed by SDS-PAGE and immunodetected with the phosphotyrosine antibodies PY20 or 4G10 and FLAG or α2-chimaerin antibodies.

Both antibodies effectively detected phosphotyrosine, but because the PY20 antibody had a generally higher background and strong non-specific detection of size markers (Figure 3.10), 4G10 phosphotyrosine antibody was used subsequently.

3.4 Identification of Tyrosine Phosphorylation

To test whether any of the mutated tyrosines are likely candidates for Fyn phosphorylation, α2-chimaerin tyrosine mutants and Fyn (or kinase dead Fyn) were co-transfected into COS-7 or N1E-115 neuroblastoma cells and treated with PMA to fully activate α2-chimaerin. α2-Chimaerin was affinity purified onto FLAG antibody beads and samples analysed via western blotting using the phosphotyrosine antibody 4G10.

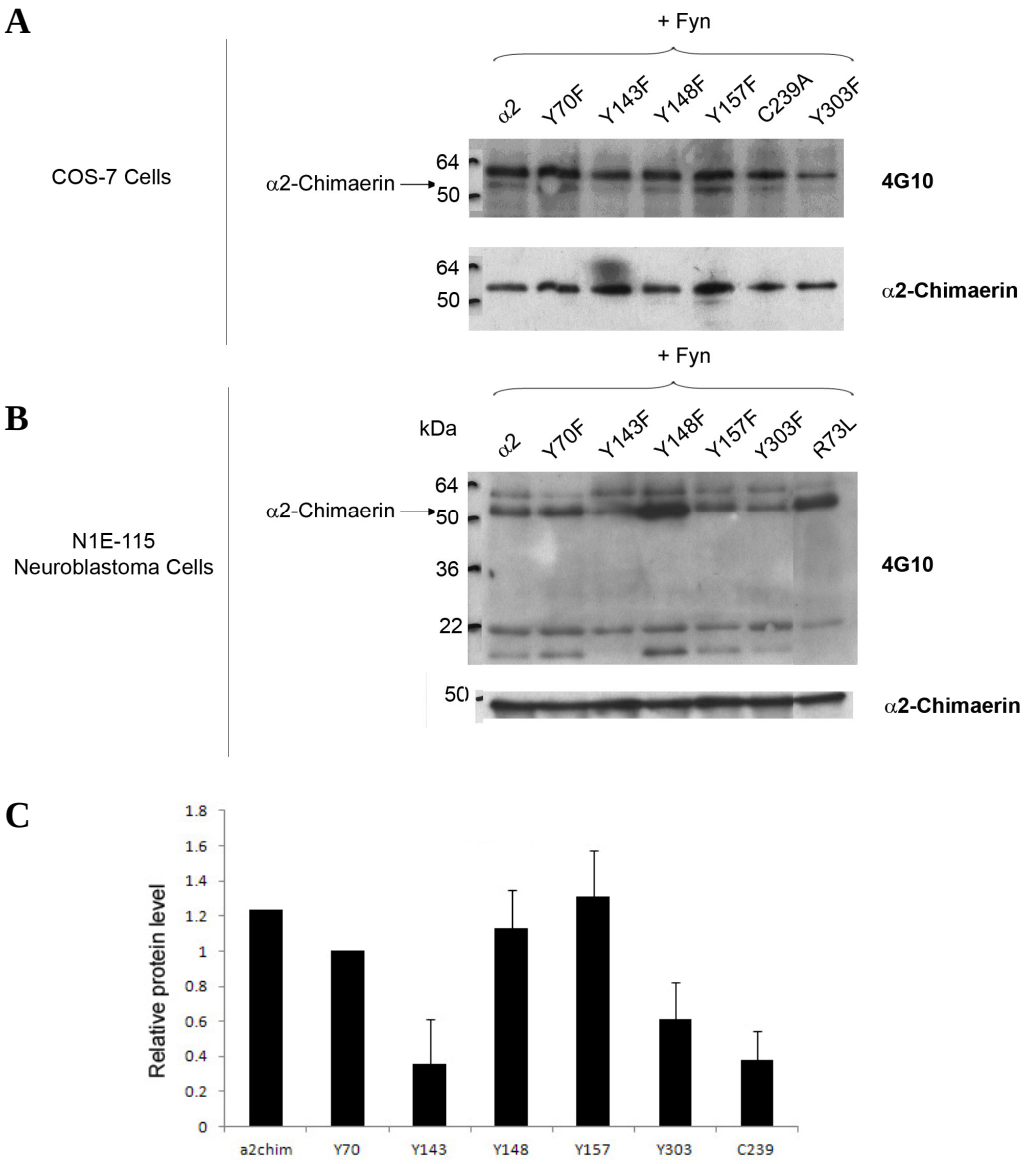


Figure 3.11 α2-Chimaerin mutants affect tyrosine phosphorylation following PMA treatment in COS-7 cells and N1E-115 neuroblastoma cells
A: COS-7 cells and **B:** N1E-115 neuroblastoma cells, were transfected with FLAG wild type or mutant α2-chimaerin and wild type Fyn and treated for 1 h with 1 µM PMA. Cells were lysed and incubated with FLAG antibody beads to pull down α2-chimaerin. Samples were analysed by SDS-PAGE and immunodetected with phosphotyrosine 4G10 and α2-chimaerin antibodies.
C: Analysis of three western blots as shown in 3.11.B of N1E-115 neuroblastoma cells expressing the various chimaerin mutants and treated with PMA using UN-SCAN-IT gel analysis software to quantitate pixels. Phosphotyrosine bands were normalised to the corresponding α2-chimaerin expression level and then expressed relative to α2-chimaerin Y70 for each experiment (normalised to 1 relative protein level), as this had more stable expression over all the experiments. Error bars represent standard deviation from the mean.

In COS-7 cells, α2-chimaerin tyrosine phosphorylation was much less than that of the upper band (Fyn). Tyrosine phosphorylation of α2-chimaerin Y143F and Y303F appeared decreased relative to wild type α2-chimaerin (Figure 3.11 A). Also, in N1E-115 cells treated with PMA, Y143F and Y303F mutants were less phosphorylated (Figure 3.11B). These experiments were repeated on three occasions and immunoblots quantified using UN-SCAN-IT gel analysis software, where pixels provide a measure of relative protein or phosphorylation levels. In these experiments very little tyrosine phosphorylation of α2-chimaerin C239A was detected, probably because the α2-chimaerin C239A mutant is not 'opened up' by PMA (see Figure 3.1) and therefore tyrosine residues remain obscured from Fyn.

3.5 Semaphorin 3A Treatment of N1E-115 Neuroblastoma Cells Expressing Chimaerin Mutants

To investigate tyrosine phosphorylation in sema 3A signalling, N1E-115 neuroblastoma cells were co-transfected with α2-chimaerin tyrosine mutants and Fyn (or kinase dead Fyn) and treated with sema 3A. α2-Chimaerin was affinity purified onto FLAG antibody beads and samples analysed via western blotting using the phosphotyrosine antibody 4G10.

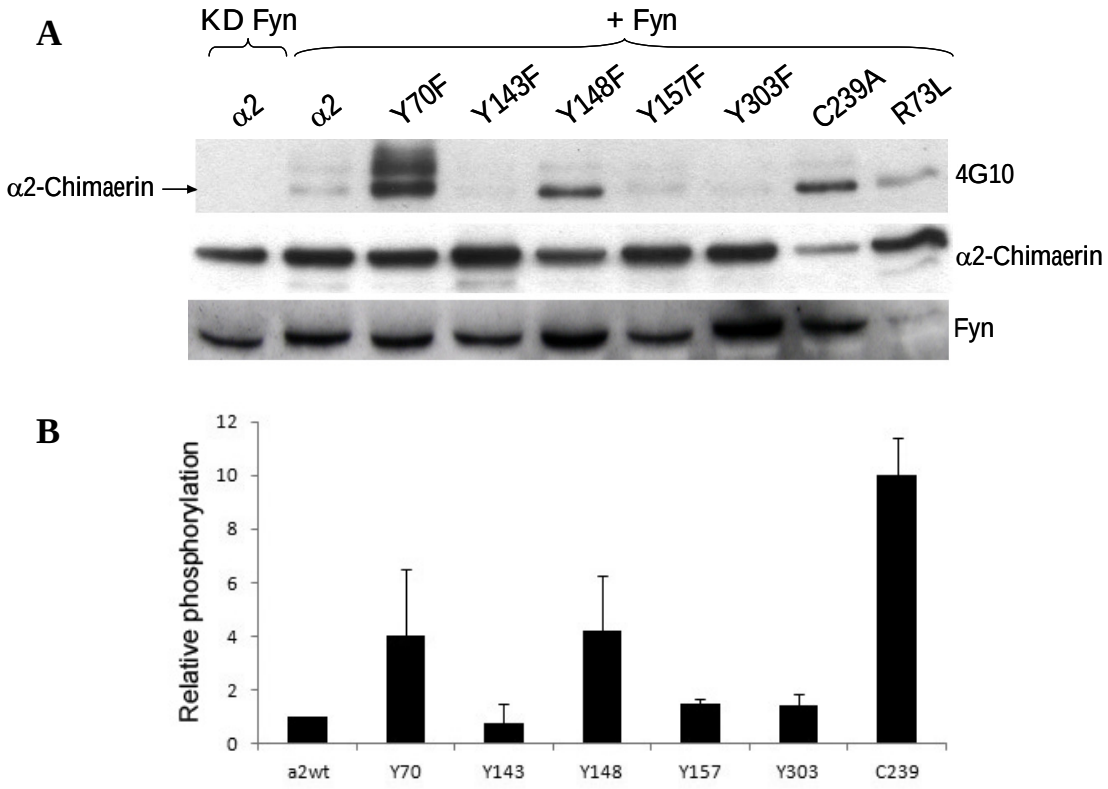


Figure 3.12 α2-Chimaerin mutants affect tyrosine phosphorylation after sema 3A treatment of N1E-115 neuroblastoma cells A: N1E-115 neuroblastoma cells were transfected with Flag-wild type or mutant α2-chimaerin and wild type or kinase dead Fyn. Cells were treated for 10 minutes with sema 3A. α2-Chimaerin was affinity purified onto FLAG antibody beads. Samples were analysed by SDS-PAGE and western blotted with phosphotyrosine 4G10, Fyn and α2-chimaerin antibodies. **B:** Analysis of three of the above experiments western blots using UN-SCAN-IT gel analysis software. Phosphotyrosine values were measured relative to their respective α2-chimaerin signal and expressed relative to α2-chimaerin wild type protein level (1) in each experiment. Error bars represent standard deviation from the mean.

In sema 3A treated cells, tyrosine phosphorylation of wild type α2-chimaerin was very slight (Figure 3.12, lane 2), but Y70F, Y148F and C239A, were more strongly phosphorylated. There was very slightly decreased tyrosine phosphorylation of the

Y143F and Y303F mutants compared with wild type α2-chimaerin detectable only in some experiments (Figure 3.12A). There was no phosphorylation when kinase dead Fyn was co-transfected with α2-chimaerin (Figure 3.12 A). These data suggest that α2-chimaerin tyrosines 143 and possibly 303 are potential sites of phosphorylation in sema 3A treated cells expressing Fyn. Increased tyrosine phosphorylation may be due to intramolecular interactions being affected by the tyrosine mutations. In contrast to figure 3.11, where PMA stimulation of α2-chimaerin C239A mutant resulted in less phosphorylation, sema 3A stimulation caused increased phosphorylation when compared to wild type α2-chimaerin. As shown in figure 3.1, the C239A mutant cannot be activated by PMA. This demonstrates that α2-chimaerin activation is a complex process and suggests that there are mechanisms other than through the C1 domain for α2-chimaerin activation and phosphorylation.

3.6 Ephrin A1 Treatment of N1E-115 Neuroblastoma Cells Permanently Expressing EphA4

α2-Chimaerin interacts with the EphA4 receptor (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007; Shi *et. al.*, 2007) and Src family kinases are involved downstream of ephrin/EphA4 signalling and can bind EphA4 (Ellis *et. al.*, 1996). N1E-115 neuroblastoma cells did not respond to ephrin A1 treatment and detectable EphA4 was not present in these cells in western blots (results not shown). An N1E-115 neuroblastoma cell line permanently expressing EphA4 receptor was therefore produced by transfection with EphA4 pcDNA3 and G418 selection. This cell line permanently expressing EphA4, was used to investigate Fyn phosphorylation of α2-chimaerin by

transient co-transfection with Fyn and FLAG-α2-chimaerin mutants. Cells were treated with pre-clustered ephrin A1 for ten minutes before lysis. Chimaerin was affinity purified onto FLAG antibody beads, samples were analysed by SDS-PAGE and immunoblotted with the 4G10 antibody to examine phosphotyrosine levels.

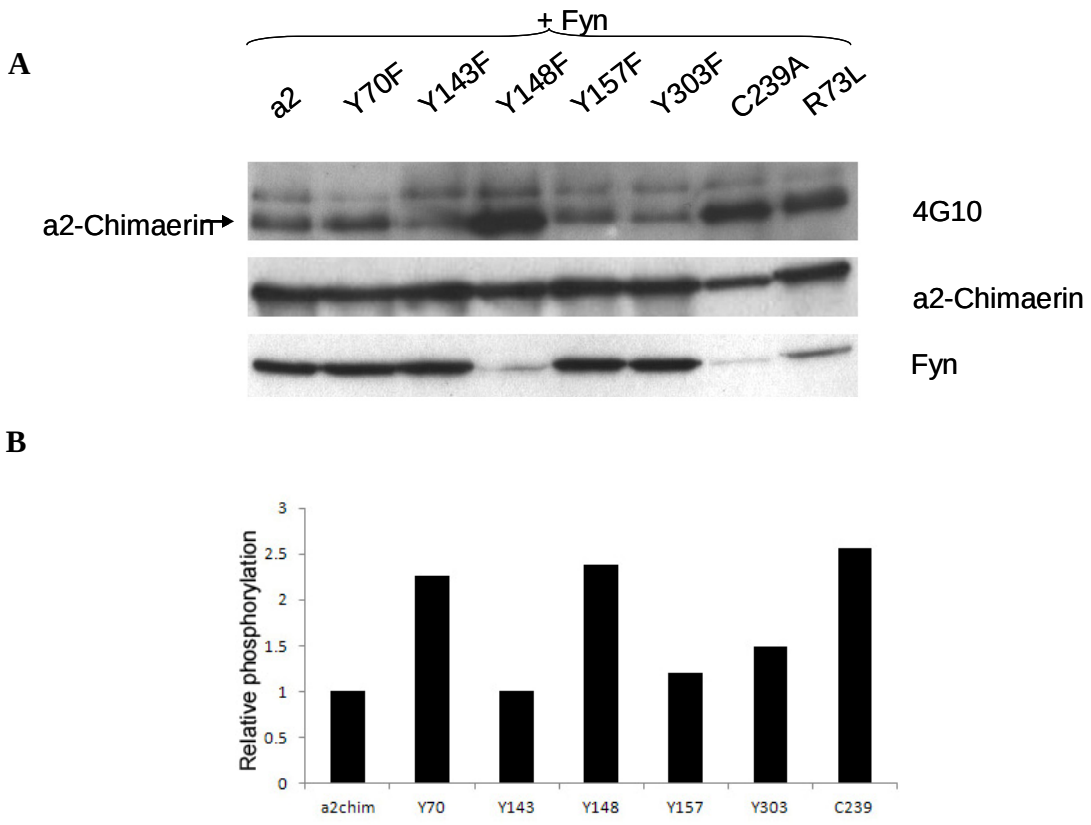


Figure 3.13 α2-Chimaerin mutants affect tyrosine phosphorylation downstream of ephrinA1/EphA4 signalling A: N1E-115 neuroblastoma cells permanently expressing EphA4 receptor were transfected with FLAG-tagged α2-chimaerin and mutants plus wild type Fyn. Cells were treated for 10 minutes with pre-clustered ephrin A1 before being lysed and incubated with FLAG antibody beads to pull down α2-chimaerin. Samples were immunodetected with phosphotyrosine 4G10, Fyn and α2-chimaerin antibodies. This experiment was repeated twice. **B:** Analysis of one experiment was performed using UN-SCAN-IT gel analysis software. Phosphotyrosine values were measured relative to their respective α2-chimaerin signal and expressed relative to α2-chimaerin wild type protein level (1).

Figure 3.13 shows tyrosine phosphorylation of α2-chimaerin mutants occurred to different extents in ephrin A1/ EphA4 signalling. The Y148F, C239A and possibly Y70F

mutants showed increased tyrosine phosphorylation compared with wild type α2-chimaerin. The R73L mutant was highly phosphorylated in some experiments (up to 10 times higher than wild type) therefore it was not included in UN-SCAN-IT analysis shown in figure 3.13. This could be due to alterations in the SH2 domain caused by the R73L mutation allowing more access for the kinase to phosphorylate. Y143F and Y303F showed less phosphorylation than wild type, although not reproducibly in the N1E-115 neuroblastoma cells permanently expressing EphA4. Several other tyrosine mutants showed enhanced phosphorylation. Increased tyrosine phosphorylation of some mutants is more difficult to explain (see Discussion, Chapter 6.0). However, phosphorylation of tyrosine 143 and 303 mutants appeared reduced to some extent in the three cell lines tested (N1E-115, N1E-115 expressing EphA4 and COS-7) irrespective of activation (sema 3A, ephrin A1 or PMA, respectively), and consistently when PMA treated (Figure 3.11B). These sites appear to be candidates for Fyn phosphorylation when chimaerin is activated/translocated.

3.7 Cell Morphology

Results in this chapter indicate that α2-chimaerin Y143 is a Fyn site in PMA treated cells, but other tyrosines may also be phosphorylated in *in vivo* signalling, as suggested by Shi *et. al.*, 2007 and Wegmeyer *et. al.*, 2007. Since α2-chimaerin influences the morphology of neurones (Buttery *et. al.*, 2006), we investigated whether tyrosine mutations altered α2-chimaerin function. α2-Chimaerin mutants were transfected into primary hippocampal neurones in culture isolated from day 18 rat embryos. After 3-5 div neurones were stained with axonal marker Tau antibody, phalloidin and α2-chimaerin antibody, and

imaged by confocal microscope. Sholl analysis was used to quantify these results (Sholl, 1953). This was developed for analysis of the dendritic organisation of cat visual and motor cortices (Sholl, 1953). Concentric circles are drawn around the centre of the cell soma at fixed step radii. The number of intersections of cell processes crossing the circle is counted. The data plotted as the number of intersections, versus the distance from the cell soma. For this study, circles at 2 µm intervals between 0 and 250 µm were used. A 0.5 µm radius span around each radius value was used as a margin to make continuous measurements of the intersections and the median calculated for each radius step (Figure 3.14).

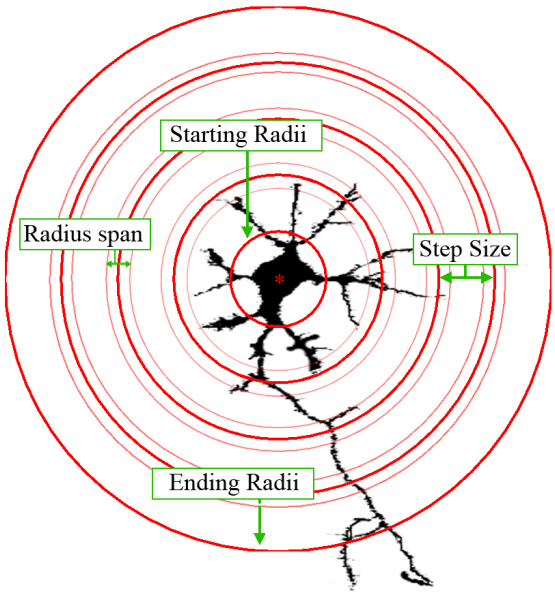
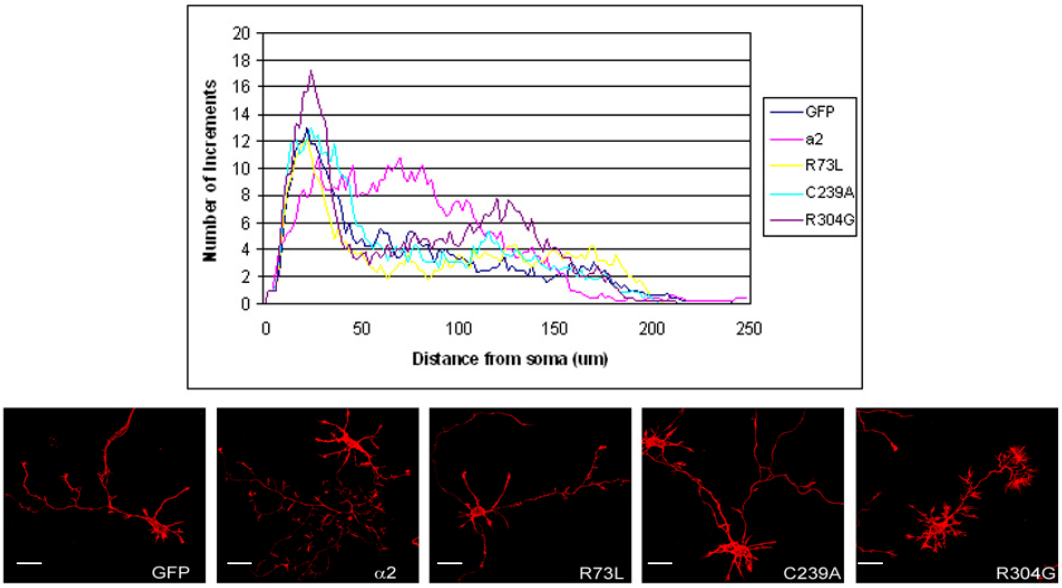


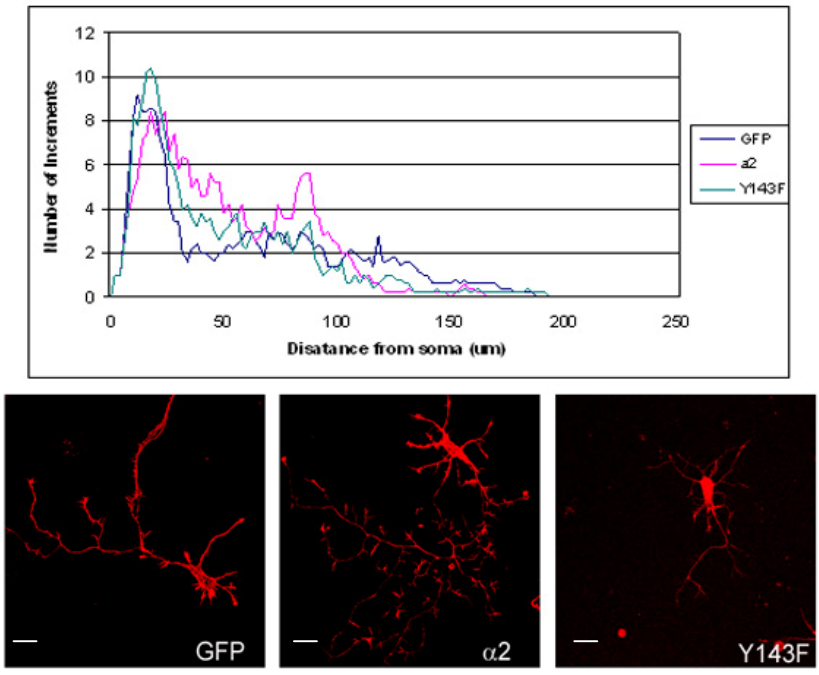
Figure 3.14 Diagramatic representation of Sholl analysis A series of concentric circles were drawn around the centre of the cell soma with a fixed step between them. The number of times the radii is interrupted by a cell process was counted, the radius span represents the margin where continuous measurements of intersections were made and the median calculated.

Figure 3.15A-D shows typical examples of the controls and mutant α2-chimaerin transfected neurones and the quantification of these results by Sholl Analysis.

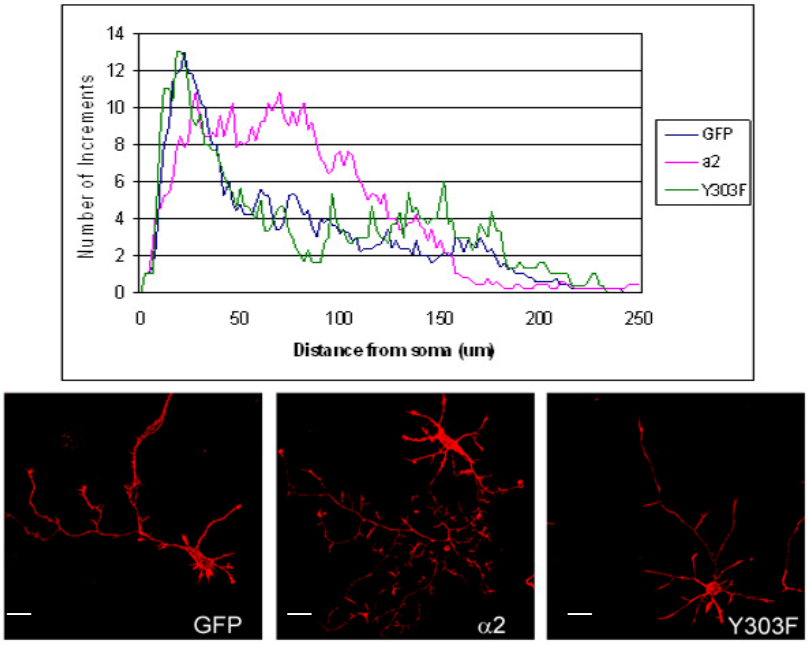
(A) Domain Inactivating Mutants



(B) Fyn Site α2-Chimaerin Y143F Mutant



(C) Potential Fyn Site α2-Chimaerin Y303F Mutant



(D) Other Phosphotyrosine Mutants

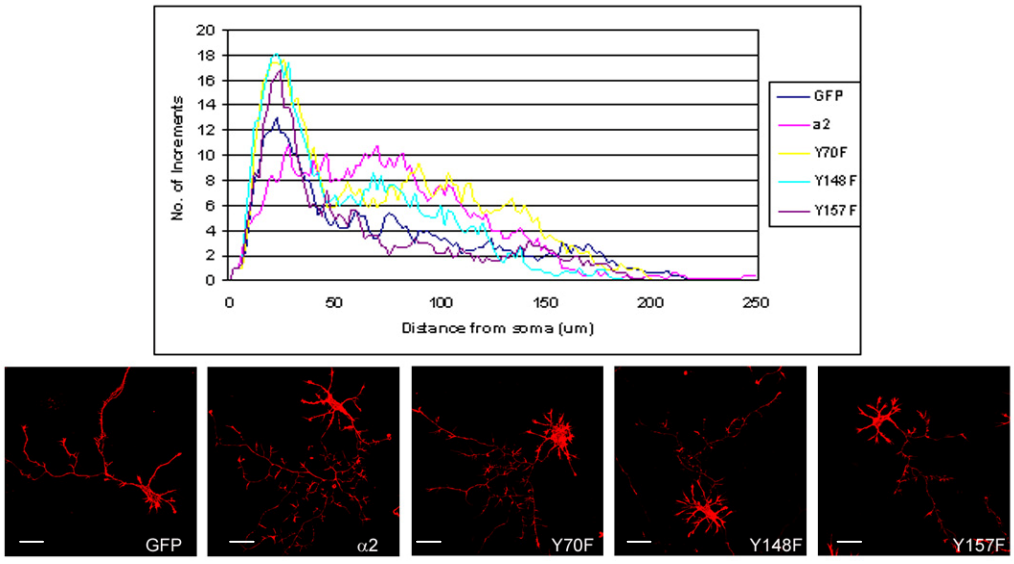


Figure 3.15A-D: α2-Chimaerin mutants affect morphology of developing hippocampal neurones E18 primary hippocampal neurones were transfected with

Flag wild type or mutant α2-chimaerin cDNAs. Cells were fixed with 4 % paraformaldehyde and stained with TRITC-labelled phalloidin (red). A representative picture for GFP, α2-chimaerin wild type and each of the α2-chimaerin mutant transfected cells is shown for each graph. Scale bars represent 10 µm. Graphs show the average of five cell images per GFP, α2-chimaerin wild type or mutant, analysed by Sholl analysis. **(A)** GFP, α2-chimaerin wild type and the three domain inactivating α2-chimaerin mutants. **(B)** GFP, α2-chimaerin wild type and Y143F mutant. **(C)** GFP, α2-chimaerin wild type and Y303F mutant. **(D)** GFP, α2-chimaerin wild type and three tyrosine mutants; Y70F, Y148F and Y157F.

Results showed that FLAG-tagged α2-chimaerin electroporated into E18 hippocampal neurones had less dendrites around the cell body, and more branchy axons compared to GFP control electroporated neurones (Figure 3.15 A). Expression of α2-chimaerin with single point mutations that inactivate the SH2 domain and C1 domain of α2-chimaerin, R73L and C239A respectively, both produced similar hippocampal neurone morphology to the GFP transfected control, suggesting these mutations prevent the function of α2-chimaerin. The α2-chimaerin R304G mutant which lacks GAP activity appeared to produce more branching on the processes around the cell body and more branching further down the processes and spread growth cones, also suggesting that the GAP function of α2-chimaerin restricts outgrowth (Figure 3.15A).

Expression of tyrosine mutants α2-chimaerin-Y143F and Y303F, which are potential Fyn sites, shown in previous experiments (Figures 3.11-3.13) resulted in similar hippocampal morphology to the GFP control (rather than to wild type α2-chimaerin morphology) (Figure 3.15B and C). Conversely, expression of tyrosine mutants α2-chimaerin-Y70F, Y148F and Y157F, resulted in much more branching around the cell body, however, further down the processes, Y157F appeared to have similar morphology to the GFP control and Y70F and Y148F appear to have more similar morphology to wild type α2-chimaerin (Figure 3.15D).

Whilst investigating the morphologies resulting from α2-chimaerin mutants, it was observed that α2-chimaerin over-expression promotes the formation of multiple axons (Figure 3.16). E18 hippocampal neurones over-expressing GFP control or α2-chimaerin or α2-chimaerin Y157F or Y143F were quantified.

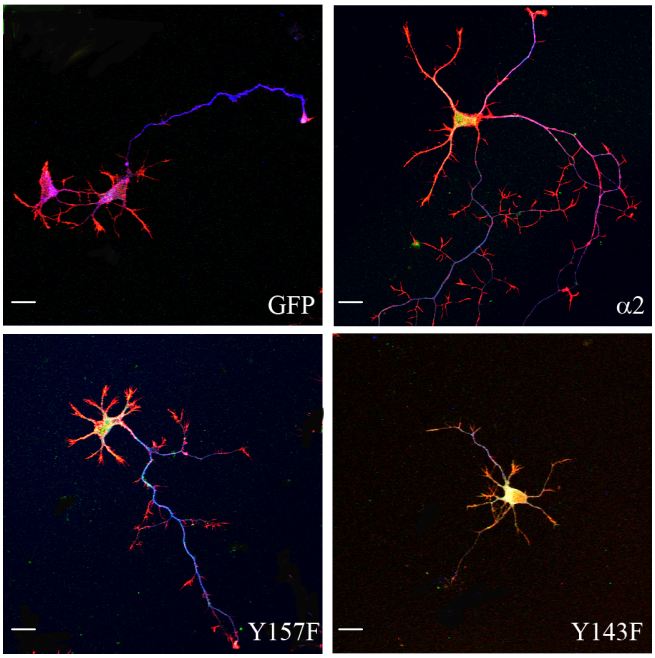


Figure 3.16 (A) α2-Chimaerin over-expression increases axon number in developing hippocampal neurones E18 primary hippocampal neurones were transfected with a GFP control or FLAG-tagged wild type and tyrosine mutant α2-chimaerin cDNAs. Cells were fixed with 4 % paraformaldehyde and stained with Cy5-mouse-anti-tau (blue), FITC-rabbit-anti-α2-chimaerin antibodies (green) and TRITC-labelled phalloidin (red). Scale bars represent 10 µm.

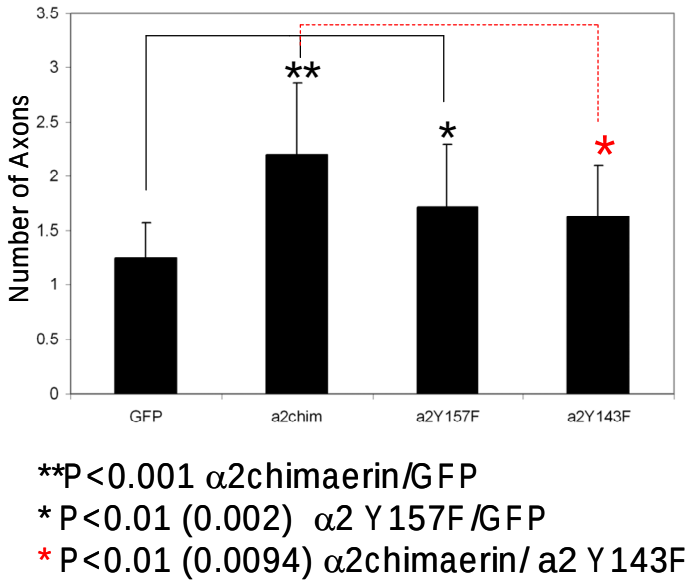


Figure 3.16 (B) α2-Chimaerin significantly increases axon number in developing hippocampal neurones compared to a GFP control The number of axons (Tau positive) were quantified from E18 primary hippocampal neurones over-expressing a GFP control or FLAG-tagged α2-chimaerin or FLAG-tagged α2-chimaerin mutants Y157F or Y143F. Error bars represent standard deviation from the mean.

Results showed a significant increase in the number of axons produced by E18 primary hippocampal neurones over-expressing α2-chimaerin compared to neurones expressing a GFP control (Figure 3.16, A and B). Phosphotyrosine mutants had a smaller difference in axon number and α2-chimaerin Y143F mutant is significantly different from α2-chimaerin. Figures 3.15 and 3.16 showed that single point mutations of α2-chimaerin are sufficient to affect the morphology of developing hippocampal neurones.

3.8 Summary

The results in chapter 3.0 showed that tyrosine phosphorylation of α2-chimaerin can occur in the sema 3A and ephrin A1/EphA4 pathways, which are important in neuronal development. It has been reported that α2-chimaerin can be phosphorylated by SFKs (Shi et. al., 2007). Chapter 3.0 has demonstrated α2-chimaerin phosphorylation by Fyn downstream of activators PMA, sema 3A and ephrin A1. Chapter 3.0 also shows that Fyn interacts with α2-chimaerin GAP domain. Single point tyrosine mutations of α2-chimaerin suggest that Y143 in the SH2-C1linker region (and possibly Y303) are possible Fyn phosphorylation sites. The equivalent β2-chimaerin tyrosine, Y153, is buried in the inactive β2-chimaerin structure (Canagarajah *et. al.*, 2004), therefore α2-chimaerin activation must occur before it can be accessed by Fyn for phosphorylation. Similarly, α2-chimaerin C239A mutant was only phosphorylated in cells treated with upstream receptor ligands, when PMA was not the sole activator of α2-chimaerin, also suggesting another route of activation. The treatment of cells with PMA and certain mutants (Y70F, Y148F, C239A and R73L) increased tyrosine phosphorylation of α2-chimaerin with sema 3A or ephrin treatment, suggesting altered accessibility by disruption of α2-chimaerin structure or conformation, or affecting other interactions. Additionally, the degree of α2-chimaerin activation that can be achieved in N1E-115 neuroblastoma cells was not particularly high, it may be that neurones would be a more physiological reporter system.

Neurone morphology studies in this chapter suggest that over-expressed single point mutations of α2-chimaerin are sufficient to produce morphological changes of hippocampal neurones. Over-expression of α2-chimaerin wild type and tyrosine mutants

Y70F, Y148F and Y157F appear to increase neurite branching, particularly Y70 and Y148 mutants which were also capable of enhanced Fyn phosphorylation. Whereas, α2-chimaerin domain inactivating mutants along with α2-chimaerin Y143F and Y303F mutants showed similar hippocampal neurone morphology to the GFP control. This suggests that phosphorylation of Y143 and Y303 (likely by Fyn) could regulate α2-chimaerin activity in neurones. It has been reported that PKC phosphorylation of serine 169 in β2-chimaerin is involved in its regulation (Griner *et. al.*, 2010). As this region of α2-chimaerin differs slightly from β2-chimaerin (and the S169 residue is not conserved in α2-chimaerin) it is possible that they are regulated in slightly different ways.

Chapter 3.0 demonstrates interesting developments in α2-chimaerin tyrosine phosphorylation, but creates more questions over α2-chimaerin regulation and activation.

Chapter 4.0:

Results II:

α2-Chimaerin in the ephrin A1/EphA4 Collapse Signalling Pathway

4.1 α2-Chimaerin in the ephrin A1/EphA4 Collapse Signalling Pathway

Ephrin/Eph signalling has been implicated in a wide range of processes in neuronal development and functions in cell migration and axonal guidance. α2-Chimaerin is a key component of EphA4 forward signalling, resulting in growth cone collapse (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007; Shi *et. al.*, 2007). Results in the previous chapter showed that α2-chimaerin was tyrosine phosphorylated downstream of ephrin A1/EphA4 signalling (Figure 3.8) and that α2-chimaerin GAP domain interacts with Fyn, possibly acting as a scaffold (Figure 3.7). This part of the study aims to further investigate α2-chimaerin interactions in ephrin A1/EphA4 forward signalling.

4.2 EphA4 and α2-Chimaerin Interaction

The published literature regarding an EphA4 and α2-chimaerin interaction was not consistent in relation to the binding sites. One study reported that EphA4 interacts with the C-terminal of both α1 and α2-chimaerin (Iwasato *et. al.,* 2007), and two others showed an α2-chimaerin SH2 domain interaction with EphA4 and another possible site in the remainder of α2-chimaerin (Beg *et. al.,* 2007; Wegmeyer *et. al.,* 2007).

Chimaerin deletion mutants were used to further map the region of interaction of EphA4 with α2-chimaerin. COS-7 cells were transfected with the EphA4 receptor and either FLAG-tagged α2-chimaerin or truncated α2-chimaerin fragments. Chimaerin was purified onto FLAG antibody beads and samples were analysed by SDS-PAGE and immunoblotted with EphA4 antibody to identify the binding region.

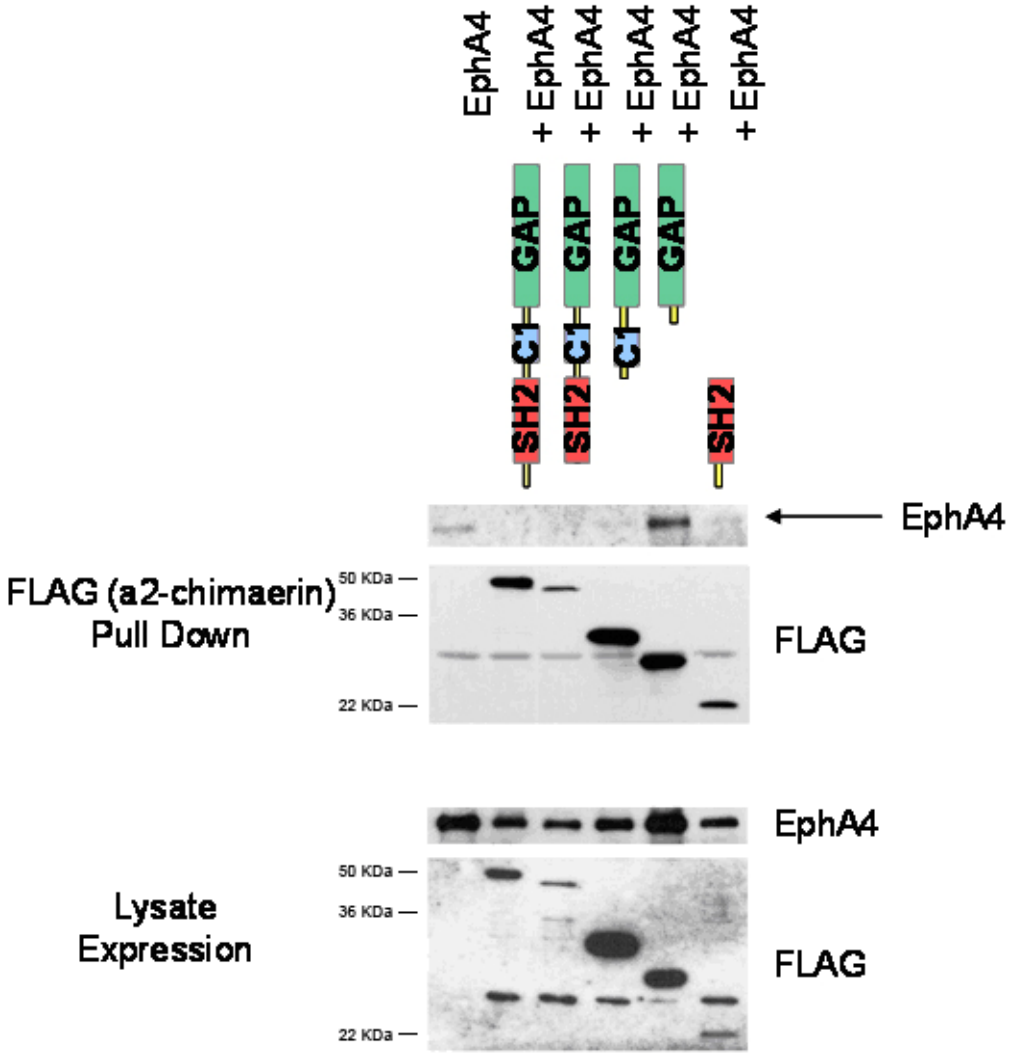


Figure 4.1 EphA4 interacts with α2-chimaerin's GAP domain COS-7 cells were transfected with FLAG-tagged α2-chimaerin or fragments and wild type EphA4. Cells were lysed and incubated with FLAG antibody beads to pull down FLAG α2-chimaerin. Samples were analysed by SDS-PAGE and immunodetected with EphA4 and FLAG antibodies. The above figure is representative of two experiments.

EphA4 interacted with the GAP domain of α2-chimaerin, but not the SH2 domain (Figure 4.1). However, a background band can be seen in the negative control lane. When cells were treated with pre-clustered ephrin A1 for 10 minutes prior to lysis to induce ephrin

A1/EphA4 forward signalling, a difference in EphA4 binding of α2-chimaerin was observed (Figure 4.2).

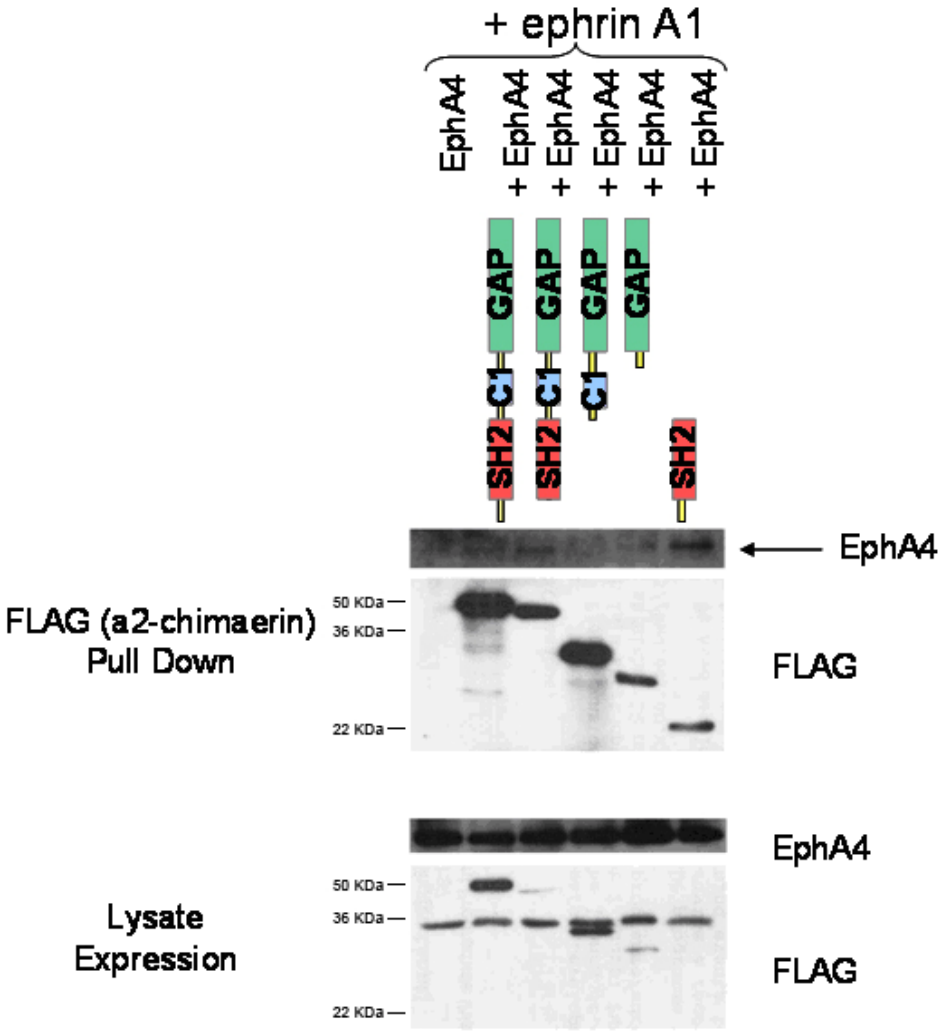


Figure 4.2 EphA4 interacts with α2-chimaerin's SH2 domain when stimulated with the ligand ephrin A1 COS-7 cells were transfected with EphA4 and FLAG-tagged α2-chimaerin or fragments. Cells were treated for 10 minutes with pre-clustered ephrin A1 before lysis and α2-chimaerin affinity purified onto FLAG antibody beads. Samples were analysed with EphA4 and FLAG antibodies. The above figure is representative of two experiments.

EphA4 bound to the SH2 domain of α2-chimaerin, although binding was quite weak, rather than to the GAP domain, which differed from the result without stimulation by ephrin A1 as shown in figure 4.1. With ephrin A1 stimulation, EphA4 also associated with α2-chimaerin 39-459 (Figure 4.2). The N-terminal deletion, 39-459, construct likely releases α2-chimaerin into an open conformation, resulting in other domains becoming more available for binding.

4.3 α2-Chimaerin Interacts with the Adaptor Protein Nck1

Nck1 is an adaptor protein with three SH3 domains and an SH2 domain. Nck interacts with RTKs including the ephrins, and links phosphotyrosine signalling to proteins involved in regulating the cytoskeleton (Bladt *et. al.,* 2003). Studies have shown Nck to interact with EphB1, EphA2 and ephrin B3 in a phosphotyrosine dependant manner via its SH2 domain (Stein *et. al.*, 1998; Miura *et. al.*, 2009; Xu and Henkenmeyer 2009). Nck1/2 knock out mice have survival defects, but mice lacking one of the two Nck genes (Nck1), with conditional knock out of Nck2 in the nervous system, have developmental defects of axonal guidance, similar to those of both EphA4 and α2-chimaerin gene deletions in mice. Nck has been shown to bind to α2-chimaerin via an SH3 domain (Fawcett *et. al.,* 2007).

To investigate the region of interaction of α2-chimaerin with Nck1, COS-7 cells were co-transfected with pXJ40 HA-Nck1 (kindly provided by E. Manser, Singapore) and FLAG-tagged α2-chimaerin or truncated FLAG-tagged α2-chimaerin fragments. Cells were untreated or treated with PMA to fully open α2-chimaerin prior to cell lysis. FLAG-

tagged α2-chimaerin constructs were affinity purified onto FLAG antibody beads and samples were analysed via western blotting as indicated in figure 4.3.

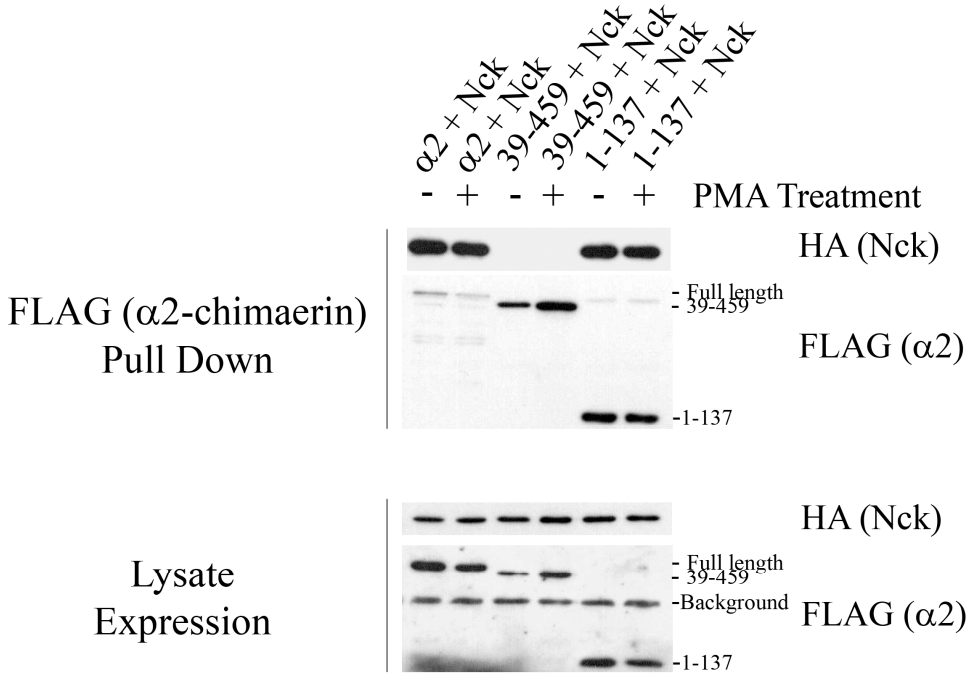


Figure 4.3: Nck1 interacts with the first 39 amino acid residues of α2-chimaerin COS-7 cells were transfected with FLAG tagged, full length or fragments of α2-chimaerin, and HA tagged Nck1. Cells were untreated or treated with 1μM PMA for 1 h before lysis and incubation with FLAG antibody beads to pull down α2-chimaerin. Samples were immunodetected with HA and FLAG antibodies. The above figure is representative of two experiments.

Results showed that Nck1 bound to α2-chimaerin 1-137 and that deletion of the N-terminal 1-39 amino acid residues of α2-chimaerin eliminates binding, suggesting that an essential binding site lies within the first 39 amino acids. Treatment with PMA had no effect on Nck1 binding α2-chimaerin (Figure 4.3), indicating that Nck can interact with both active and inactive conformations. Since full length α2-chimaerin was expressed at slightly lower levels relative to the α2-chimaerin 1-137 fragment but bound an equivalent

amount of Nck1, this result also suggests that 1-39 may not be the only region involved in binding.

4.4 Nck1 Interacts with α2-Chimaerin and Promotes Fyn Binding

Taken together, these results have shown three different regions of α2-chimaerin bind to three different protein components of the ephrin/EphA4 signalling pathway; the N-terminal of α2-chimaerin is involved in binding Nck1, α2-chimaerin SH2 domain appears to bind the EphA4 receptor following ephrin A1 stimulation, and α2-chimaerin GAP domain binds Fyn. Firstly, to determine whether the α2-chimaerin, Nck1 and Fyn could form a complex, COS-7 cells were transfected with α2-chimaerin and either Nck1 or Fyn or both. Cells were untreated or treated with PMA 1 h prior to lysis. FLAG-tagged α2-chimaerin was affinity purified onto FLAG antibody beads and proteins immunodetected as indicated in Figure 4.4.

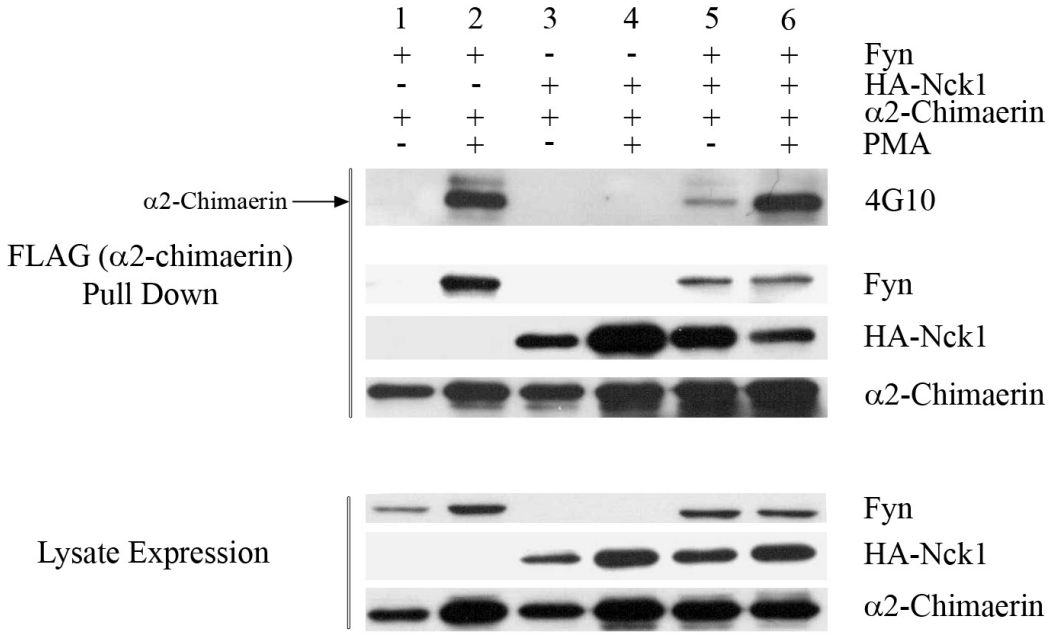


Figure 4.4: Nck1 interacts with α2-chimaerin and promotes Fyn binding COS-7 cells were transfected with combinations of FLAG-tagged α2-chimaerin and Fyn (lanes 1 and 2) or HA-tagged Nck1 (lanes 3 and 4) or with all three constructs (lanes 5 and 6). Cells were untreated (lanes 1, 3 and 5) or treated with 1μM PMA for 1 h before lysis (lanes 2, 4 and 6). FLAG-α2-chimaerin was affinity purified onto FLAG antibody beads and samples analysed with 4G10 phosphotyrosine antibody, Fyn, HA and FLAG antibodies. The above figure is representative of two experiments.

Results showed that Nck1 binding to α2-chimaerin was sufficient to allow some Fyn binding to α2-chimaerin in the absence of PMA (Figure 4.4, compare lanes 1 and 5). PMA treatment strongly promoted α2-chimaerin interaction with Fyn and its phosphorylation by Fyn (Figures 3.5, 3.8 and 4.4, lanes 1 and 2). However, figure 4.4, lanes 5 and 6 showed that when Nck1 is also present, Fyn binding α2-chimaerin is detected in the absence of PMA. Thus, Nck1 binding to the N-terminal region of α2-chimaerin could effectively open α2-chimaerin conformation (or partially relax the structure) for Fyn to bind. No enhancement in Fyn binding with the addition of PMA treatment was seen, although under these conditions there was an increase in tyrosine phosphorylation of α2-chimaerin (lanes 5 and 6). This result may also indicate that α2-chimaerin can be activated by Nck1 binding, but is further phosphorylated when cells are PMA treated.

Secondly, to explore whether Nck1 and Fyn can interact with each other, COS-7 cells were transfected with Fyn alone, Fyn and HA-tagged Nck1, or Fyn, HA-tagged Nck1 and FLAG-tagged α2-chimaerin. Cells were lysed and HA-Nck1 affinity purified onto HA antibody beads. Proteins were detected as indicated in figure 4.5.

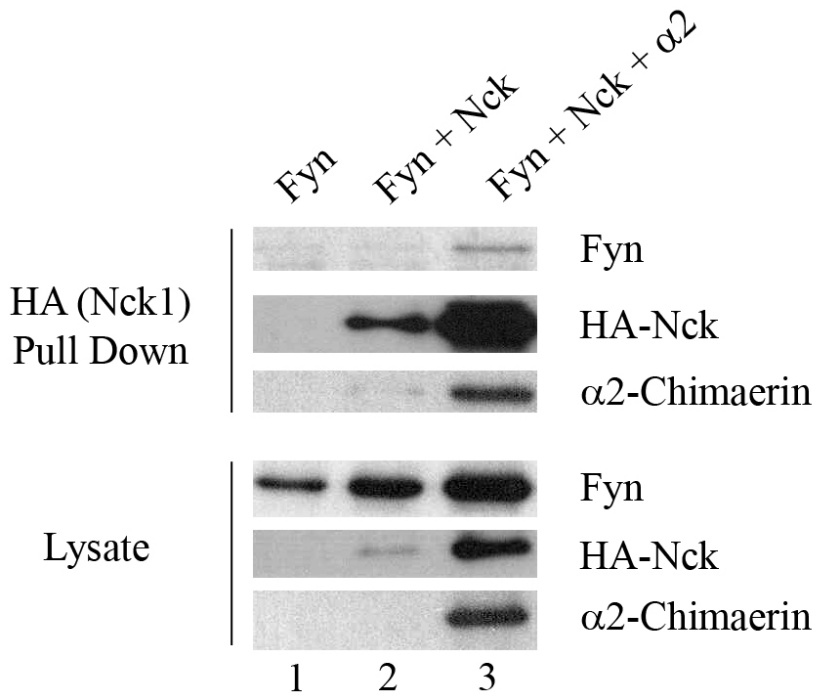


Figure 4.5: Nck1 and Fyn do not interact with each other in COS-7 cells COS-7 cells were transfected with Fyn, lane 1; Fyn and HA-tagged Nck1, lane 2, or Fyn, HA-tagged Nck1 and FLAG-tagged α2-chimaerin, lane 3. Cells were lysed and incubated with HA antibody beads to pull down HA-tagged Nck1. Samples were analysed by SDS-PAGE and immunodetected with Fyn, HA and α2-chimaerin antibodies. The above figure is representative of two experiments.

Results showed that Fyn did not pull down with HA-Nck1 alone in COS-7 cells in the absence of α2-chimaerin. When α2-chimaerin, Fyn and Nck1 were co-transfected into COS-7 cells, all three proteins could be detected in the pull down (Figure 4.5, lane 3), suggesting that α2-chimaerin acts as a scaffold to bind Fyn. However, Nck1 expression was much greater in the presence of α2-chimaerin.

Thirdly, to investigate whether Fyn can tyrosine phosphorylate Nck1 in cells, HA-Nck1 was affinity purified from COS-7 cells co-expressing Fyn or kinase dead Fyn and HA-tagged Nck1, and tyrosine phosphorylated proteins were immunodetected with the 4G10 phosphotyrosine antibody.

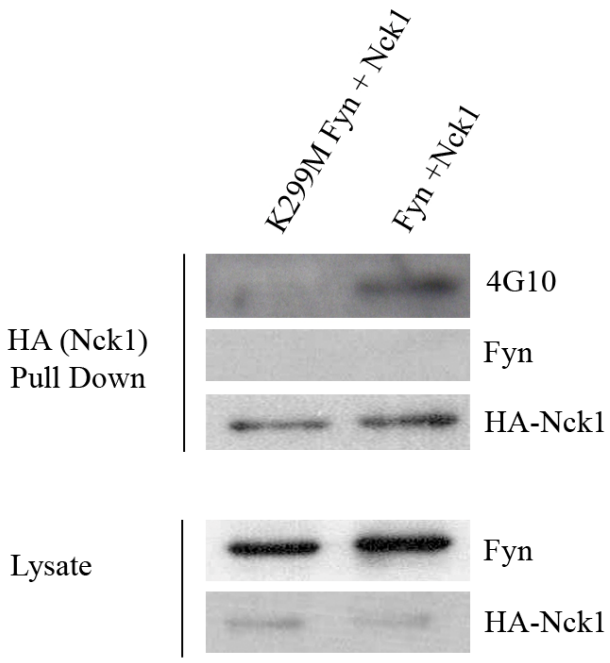


Figure 4.6: Wild type Fyn can phosphorylate Nck1 COS-7 cells were transfected with Fyn or Kinase Dead Fyn and HA-tagged Nck1. HA-tagged Nck1 was affinity purified onto HA antibody beads. Samples were analysed by SDS-PAGE and western blotted with phosphotyrosine 4G10 antibody and with Fyn and HA antibodies. The above blot is representative of three experiments.

Results showed (Figure 4.6) that in COS-7 cells, wild type Fyn tyrosine phosphorylated Nck1. No Fyn or kinase dead Fyn pulled down with HA-Nck1. Figures 4.5 and 4.6 demonstrate that Fyn can phosphorylate but not bind Nck1.

These results suggest that the three protein components of the ephrin/EphA4 signalling pathway, α2-chimaerin, Fyn and Nck1 could potentially form a complex, with α2-chimaerin acting as a scaffold for the other two proteins.

4.5 α2-Chimaerin in the ephrin A1/EphA4 Signalling Pathway

4.5.1 α2-Chimaerin shRNA

To further investigate the ephrinA1/EphA4 signalling pathway, a shRNA approach was also undertaken. ShRNA for α2-chimaerin was produced as described in Chapter 2 Methods. The system used was siSTRIKE U6 Hairpin Cloning System from Promega. To test the α2-chimaerin shRNA, N1E-115 neuroblastoma cells were transfected with the shRNA sequences for α2-chimaerin or the vector or scrambled sequence controls. G418 at 800 µg/ml selection was added the next day to select for cells expressing the psiSTRIKE vector and the cells lysed five days later. Samples were analysed by SDS-PAGE and immunoblotted with α2-chimaerin antibody and actin antibody control.

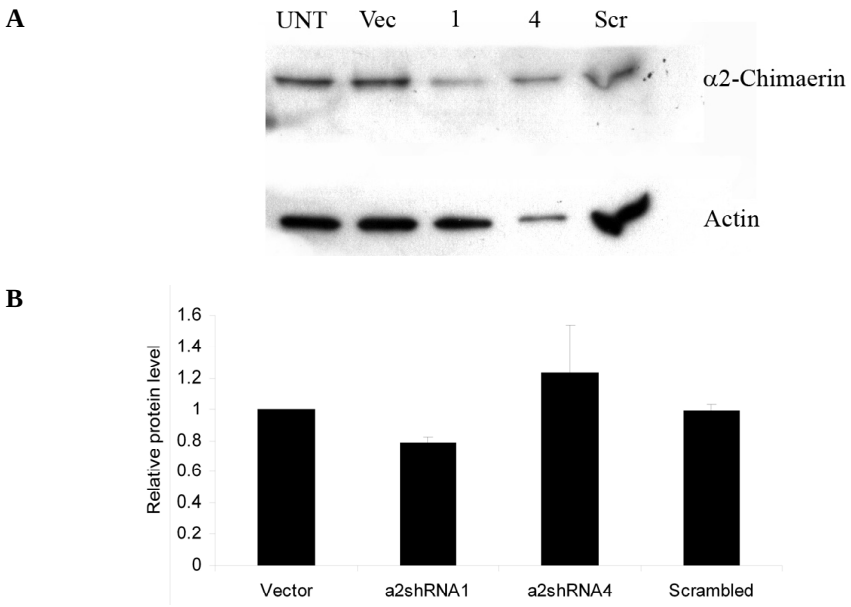


Figure 4.7: α2-Chimaerin shRNA sequence 1 knocks down α2-chimaerin N1E-115 neuroblastoma cells were untransfected or transfected with vector or scrambled shRNA controls or α2-chimaerin shRNA sequences 1 or 4. Cells were treated with 800 µg/ml G418 selection 24 hours later. Cells were lysed after five days and samples immunodetected with α2-chimaerin and actin antibodies (**A**). The blots from two experiments were subjected to UN-SCAN-IT analysis, where pixels are measured representing protein level. Protein level was then normalised to actin and then the vector control

(set to 1). The experiment was repeated on two occasions, error bars represent variation from the mean (**B**).

Figure 4.7 shows α2-chimaerin shRNA sequence 1 knocks down α2-chimaerin by up to 30 % (range 20-30 %). α2-Chimaerin shRNA sequence 4 does not appear to knock down α2-chimaerin when normalised to actin. There were consistently less cells and therefore less actin with expression of shRNA sequence 4.

The N1E-115 neuroblastoma cell line permanently expressing the EphA4 receptor described in Chapter 3 was utilised to investigate α2-chimaerin in ephrin A1/EphA4 signalling. Following 3-4 hours of serum starvation, N1E-115 neuroblastoma cells permanently expressing EphA4 adopted a spread out/flattened morphology. Following ten minute treatment of pre-clustered ephrin A1, the cells round up (Figure 4.8).

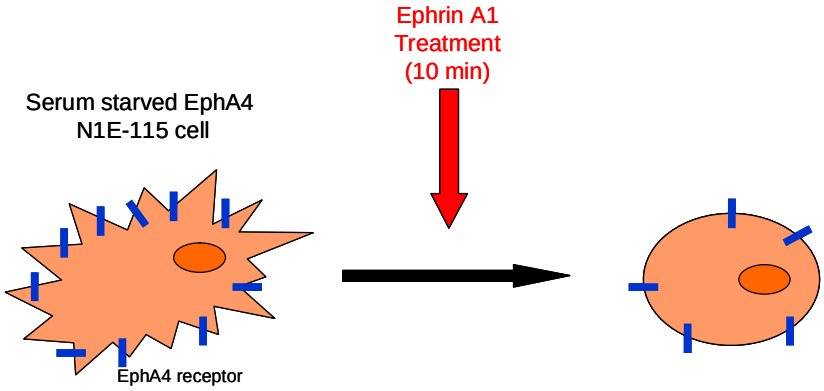


Figure 4.8 Schematic of N1E-115 neuroblastoma cells permanently expressing the EphA4 receptor before and after pre-clustered ephrin A1 treatment.

4.5.2 EphA4 expressing N1E-115 Neuroblastoma Cell Collapse Assay

It was investigated whether the N1E-115 neuroblastoma cell line permanently expressing EphA4 could be used in an assay for chimaerin involvement in ephrin A1 stimulated cell collapse. Cells were untransfected or transfected with α2-chimaerin shRNA sequence 1 or control shRNA cloned in a psiSTRIKE vector expressing hygromycin resistance (see Methods) since G418 is used to maintain selection of the EphA4 cell line. Hygromycin selection was added at 400 µg/ml to the transfected cells the following day to select for cells expressing the psiSTRIKE shRNA vector. (N1E-115 neuroblastoma cells were found to be more sensitive to hygromycin compared to G418, therefore a lower concentration was required for selection). Five days later cells were serum starved for up to 4 hours to induce cell flattening and neurite formation prior to treatment with pre-clustered ephrin A1. After fixation and phalloidin staining, cell morphology of flattened or rounded cells was quantified.

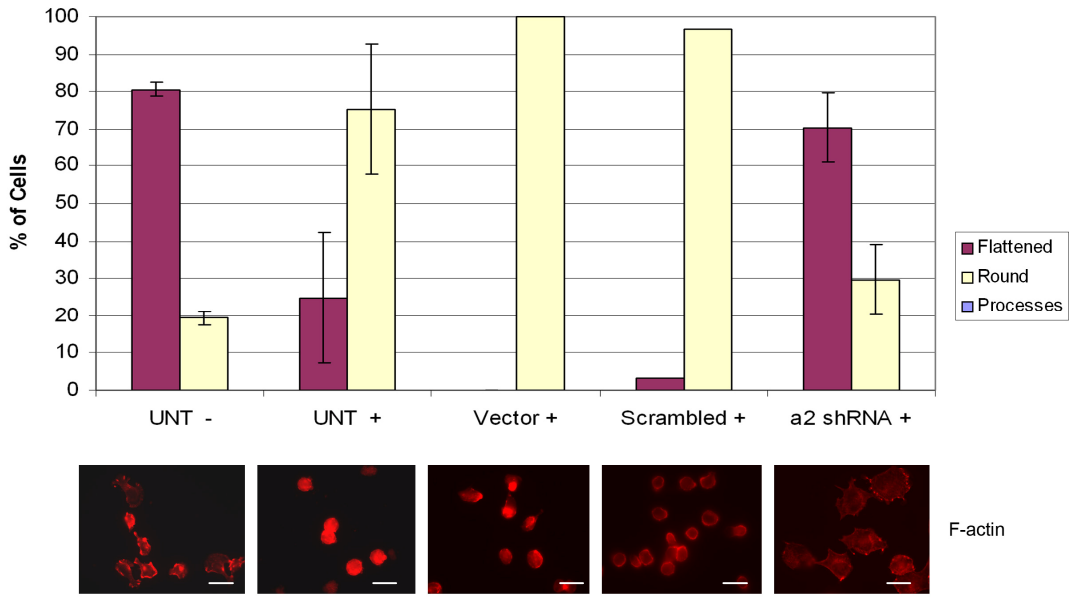


Figure 4.9 α2-chimaerin shRNA blocks cell rounding of N1E-115 neuroblastoma cells permanently expressing the EphA4 receptor N1E-115 neuroblastoma cells permanently

expressing the EphA4 receptor were either untransfected or transfected with shRNA controls, vector and scrambled or the α2-chimaerin shRNA knockdown sequence 1. The following day 400 µg/ml hygromycin selection was added, five days later, cells were untreated or treated for 10 minutes with pre-clustered ephrin A1 prior to being fixed in 4 % PFA/PBS and stained with TRITC-phalloidin for F-actin (red). 300 cells were counted for each group, the experiment was repeated on two occasions. Error bars represent standard deviation from the mean. Scale bars represent 10 µm.

Results showed that following serum starvation and without ephrin A1 treatment, untransfected cells have a spread and flattened morphology and when treated with ephrin A1 they become rounded up. Expression of α2-chimaerin shRNA (sequence 1) was sufficient to prevent cell rounding in the N1E-115 neuroblastoma cell line permanently expressing EphA4. Knockdown of α2-chimaerin prevented the ephrin-stimulated cell rounding process, which supports that α2-chimaerin participates in ephrin A1/EphA4 forward signalling. This also demonstrates that this cell line is a useful assay to investigate other necessary components in ephrin A1/EphA4 cell rounding.

Further analyses of domain mutants of α2-chimaerin were carried out to investigate their contribution to ephrin A1/EphA4 cell rounding. N1E-115 neuroblastoma cells permanently expressing EphA4 were transfected with wild type or the domain inactivating mutations of α2-chimaerin.

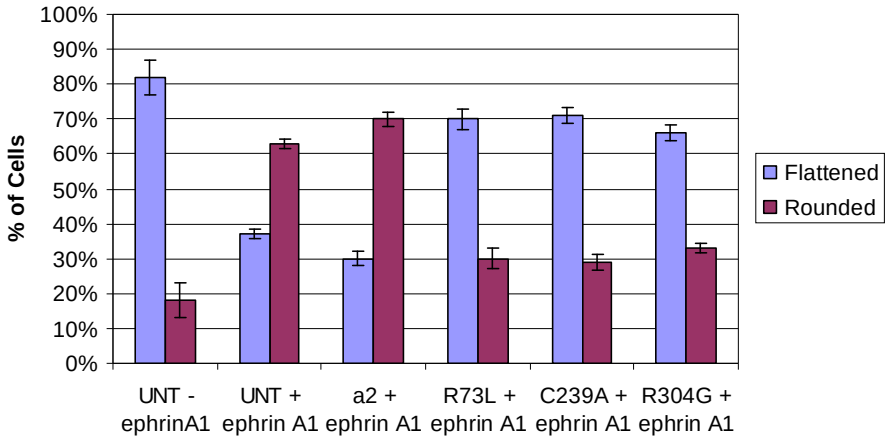


Figure 4.10: α2-Chimaerin domain inactivating mutants block cell rounding of N1E-115 neuroblastoma cells permanently expressing the EphA4 receptor N1E-115 neuroblastoma cells permanently expressing the EphA4 receptor were either untransfected or transfected with α2-chimaerin mutants. Cells were serum starved for 3-4 h and left untreated, or were treated for 10 minutes with pre-clustered ephrin A1 prior to being fixed in 4 % PFA/PBS and stained with TRITC-phalloidin for F-actin. The experiment was repeated on two occasions. Error bars show average difference. A total 300 cells were counted per treatment.

An inactivating mutation in any of α2-chimaerin domains (SH2, C1 or GAP) was sufficient to reduce cell rounding by the ligand ephrin A1 (Figure 4.10). Ideally a FLAG vector control should have been included in this experiment. Results suggest that the function of each α2-chimaerin domain is required for ephrin A1/EphA4 cell collapse.

In the N1E-115 neuroblastoma cell line permanently expressing EphA4, it was observed that α2-chimaerin was unusually found in 'dot' like structures, indicative of a possible endosomal localisation. To investigate these structures, the N1E-115 neuroblastoma cell line permanently expressing EphA4 were fixed in 4 % paraformaldehyde and stained with a series of markers for sub cellular organelles.

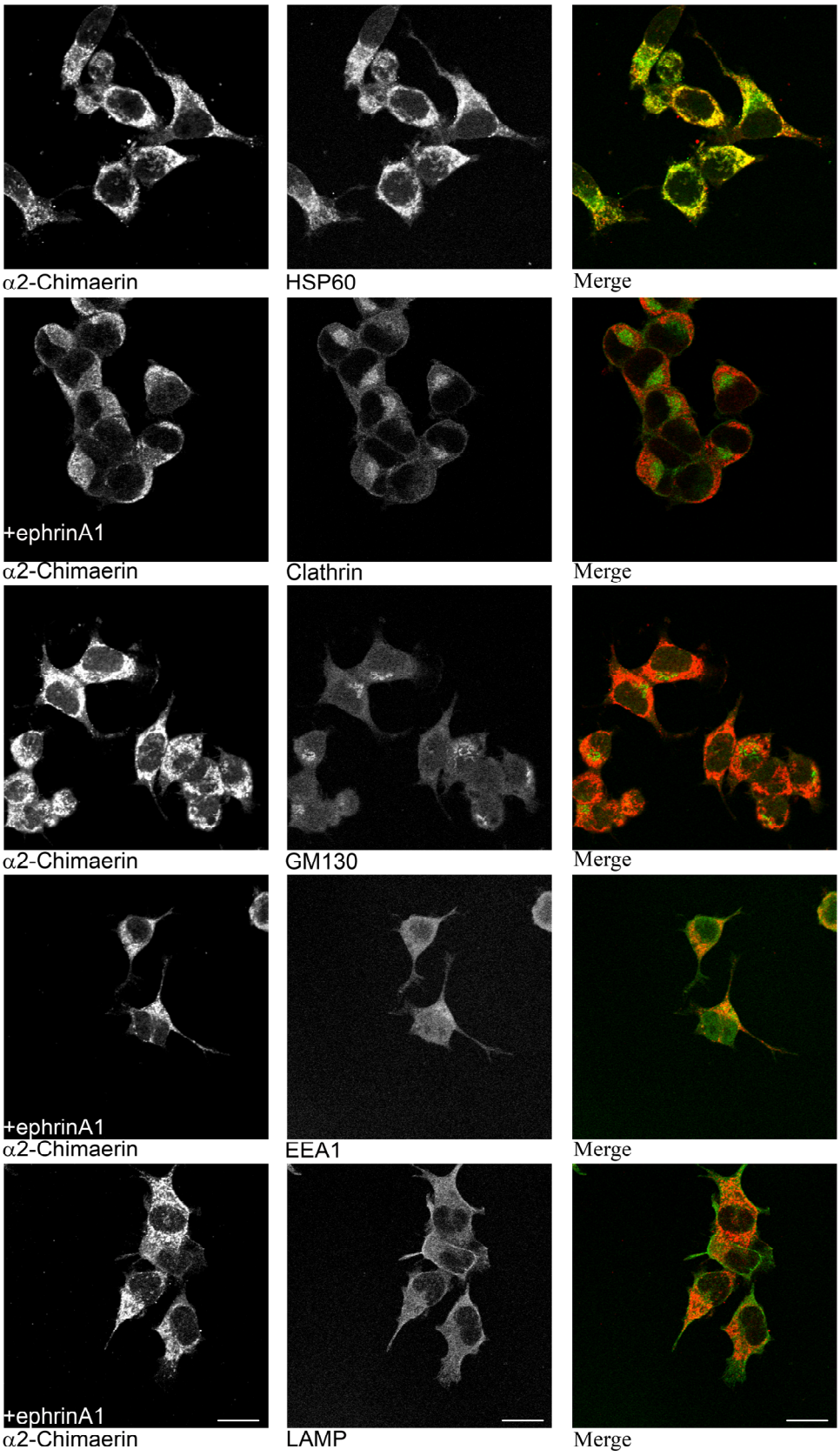

α2-Chimaerin
HSP60
Merge
+ephrinA1
α2-Chimaerin
Clathrin
Merge
α2-Chimaerin
GM130
Merge
+ephrinA1
α2-Chimaerin
EEA1
Merge
+ephrinA1
α2-Chimaerin
LAMP
Merge

Figure 4.11: α2-Chimaerin co-localises with mitochondrial matrix marker HSP60 N1E-115 neuroblastoma cells permanently expressing EphA4 receptor were untreated, or treated with pre-clustered ephrin A1, fixed with 4 % PFA and stained for α2-chimaerin with rabbit α2-chimaerin antibody and Cy5-secondary (red) antibody and FITC (green) conjugated markers were used. Scale bars represent 10 µm.

Results suggested that α2-chimaerin co-localised with the mitochondrial matrix marker HSP60 (Figure 4.11). A small amount of possible co-localisation could also be seen between α2-chimaerin and the early endosome marker EEA1 (Figure 4.11). In N1E-115 cells, over-expressed α2-chimaerin is largely cytoplasmic, although endogenous α2-chimaerin can have a particulate distribution in neurones (Hall *et. al*., 2001). N1E-115 cells permanently expressing EphA4 appear to have some constitutive activity that may affect α2-chimaerin distribution irrespective of ephrin A1 stimulation.

4.6 Summary

The results in chapter 4.0 add to the literature that emerged during this study, demonstrating that α2-chimaerin is important in the ephrin/EphA4 signalling pathway (Wegmeyer *et. al.*, 2007; Beg *et. al*., 2007; Iwasato *et. al.*, 2007; Shi *et. al*., 2007). Although knockdown was incomplete, chimaerin shRNA1 inhibited ephrin induced cell rounding in EphA4-expressing N1E115 cells. (It is also possible that there is greater chimaerin turnover in these cells with ephrin stimulation). It appears that all three α2-chimaerin domains (SH2, C1 and GAP) are required in ephrin A1/EphA4 induced response of cell rounding, as has been reported by Shi *et. al.*, 2007 (for SH2 and GAP domains). α2-Chimaerin interacts with the EphA4 receptor (Wegmeyer *et. al*., 2007; Beg *et. al*., 2007; Iwasato *et. al*., 2007), with Fyn and with Nck (Wells *et. al.*, 2006), possibly acting as a scaffold for a receptor-associated complex containing Fyn and Nck1.

However, the precise molecular interactions are not clearly established, since Fyn can also interact with EphA4 receptor via its SH2 domain (Ellis *et. al.*, 1996) and Nck SH2 could also be involved in phosphotyrosine-dependent interactions directly or indirectly receptor-associated, as with integrins (Schlaepfer *et. al.*, 1997) and ephrin B3 (Xu and Henkemeyer, 2009), whilst its SH3 domain interacts with chimaerin. α2-Chimaerin may co-localise with the mitochondrial matrix and early endosomes in the N1E-115 neuroblastoma cell line permanently expressing EphA4 receptor, in contrast to its largely cytoplasmic location in parent N1E-115 cells. This suggests that transfected α2-chimaerin may become activated and re-localize in response to EphA4 signalling (see Discussion, Chapter 6.0). Overall, this chapter has explored the protein interactions and the requirement of α2-chimaerin domains in ephrin A1/EphA4 signalling.

Chapter 5.0:

Results III:

New Interacting Partners of α2-Chimaerin

5.1 Interacting Partners of α2-Chimaerin

Known α2-chimaerin interacting partners that participate in the sema 3A pathway include Rac1, collapsin response mediator protein-2 (CRMP-2) and Cdk5/p35 (Brown *et. al.*, 2004). CRMP-2 interacts with the SH2 domain of α2-chimaerin. CRMP-2 is one of a family of proteins, CRMP-1-5 (CRMP-5 is also known as CRAM (Inatome *et. al.*, 2000; Ricard *et. al.*, 2001; Fukata *et al.*, 2002)) involved in collapse pathways, axonal outgrowth, tubulin binding and cell division (Schmidt and Strittmatter, 2007). CRMP-2 is involved in cell polarity and over-expression of CRMP-2 in neurones produces multiple axons (Inagaki *et. al.*, 2001). The Cdk5 activating molecule, p35, and Cdk5, interact with the GAP domain of α2-chimaerin and Cdk5/p35 also phosphorylates CRMP-2, priming for phosphorylation by GSK-3β (Brown *et. al.*, 2004; Cole *et. al.*, 2004; Yoshimura *et. al.*, 2005).

5.2 α2-Chimaerin Interacts with Vps28, a Member of the ESCRT-I Complex

Previously in our laboratory, using a Yeast-2 Hybrid screen, human α2-chimaerin was found to interact with human Vacuolar protein sorting 28 (Vps28) protein (C. Monfries). A Vps28 mutant in yeast shows a Class E phenotype; accumulation of vesicles consistent with early endosomes. Vps28 assembles vacuolar, endocytic and late golgi markers in endosome–like Class E compartments (Rieder *et. al.*, 1996). Vps28 is a member of the endosomal sorting complex required for transport (ESCRT)-I protein complex. The ESCRT-I complex is formed of tumour susceptibility gene 101 (TSG101) (mammalian Vps23), Vps37, Mvb12 and Vps28. The complex recognises ubiquitinated multivesicular

body (MVB) cargo through a conserved UBC (ubiquitin conjugating)-like domain in Vps23 and is required for sorting into MVB vesicles (Katzmann *et. al.,* 2001). The MVB pathway involves down regulation of active cell surface receptors and the delivery of lysosomal hydrolases degrading active receptors in the lysosome (Katzmann *et. al.,* 2001). The Vps28 gene is essential; *Drosophila* homozygous mutants are lethal and mutations in Vps28 revealed a link between Vps28 and the actin cytoskeleton (Sevrioukov *et. al.,* 2005). Null mutants of Vps28 in *Saccharomyces cerevisiae* prevent golgi to pre-vacuolar compartment transport (Hanson *et. al.,* 2002).

Vps28 interacts with the conserved C-terminal region of TSG101 (Vps23) (Bishop and Woodman, 2001). Structural studies have shown that Mvb12 subunits interact with the core region of the TSG101-Vps37 complex (Morita *et. al.,* 2007) and that ESCRT-I is assembled with the C-terminal steadiness box of Vps23 (TSG101), the N-terminal of Vps28 and the C-terminal of Vps37. The C-terminal half of Vps28 does not directly participate in the ESCRT-I complex and interacts with downstream MVB pathway components ESCRT-II and ESCRT-III factor Vps20 (Kostelansky *et. al.,* 2006; Pineda-Molina *et. al.,* 2006).

Retroviruses such as the human immunodeficiency virus type I (HIV-1) and the equine infectious anemia virus (EIAV) can interact with host cellular VPS machinery including TSG101 and Vps28 to aid particle release (Martin-Serrano *et. al.,* 2003; Tanzi *et. al.,* 2003). Vps28 is vital in MVB pathway targeting and sorting.

5.3 Vps28 Antibody Characterisation

Two mouse monoclonal antibodies were characterised. The antibodies were raised to Vps28 recombinant protein (C Monfries unpublished results) by T. Jowett (UCL Monoclonal Antibody Unit). Further studies on the two antibodies revealed that they each recognised a different half of recombinant Vps28, one antibody epitope being in the N-terminal of Vps28 and the other in the C-terminal half (C. Monfries, unpublished results). Investigation of the two antibodies was carried out to test them for further use in western blotting of brain extracts and cell staining.

Western blotting was carried out on sucrose density gradient fractions from E14 and E18 rat brains, COS-7 cell expressed HA-tagged Vps28 sample was used for comparison as a size marker (lower panel, figure 5.1).

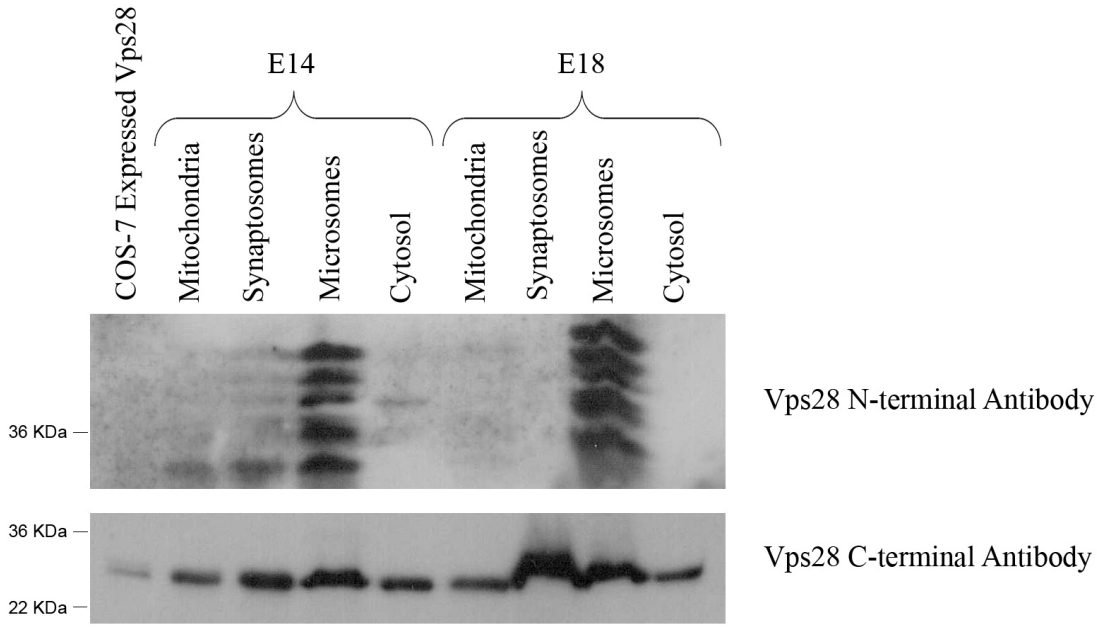


Figure 5.1 Vps28 N- and C-terminal antibody comparisons on E14 and E18 rat brain fractions Brain fractions as indicated from E14 and E18 rat brains and a COS-7 expressed Vps28 sample were analysed via SDS-PAGE. Western blotting with two different mouse monoclonal antibodies recognising the N- or C-terminal of Vps28 allowed comparison of the two antibodies.

The Vps28 antibody recognising the C-terminal, detects a single band of around 28 KDa in all fractions co-migrating with COS-7 cell expressed Vps28 (Figure 5.1). Whereas the antibody recognising the N-terminal of Vps28 detects a band of the expected size in E14 mitochondria, synaptosome and microsome fractions, although it did not seem to detect the COS-7 cell expressed Vps28. The N-terminal antibody also detected a band of the expected size in the E14 and E18 microsomal fractions, where numerous additional larger bands were also detected. The higher molecular weight bands in the microsome fractions were not further investigated, but they could possibly be ubiquitinated products. For subsequent western blotting of endogenous Vps28, the antibody recognising the C-terminal was used.

Both Vps28 antibodies were also tested for endogenous cell staining in N1E-115 cells permanently expressing EphA4.

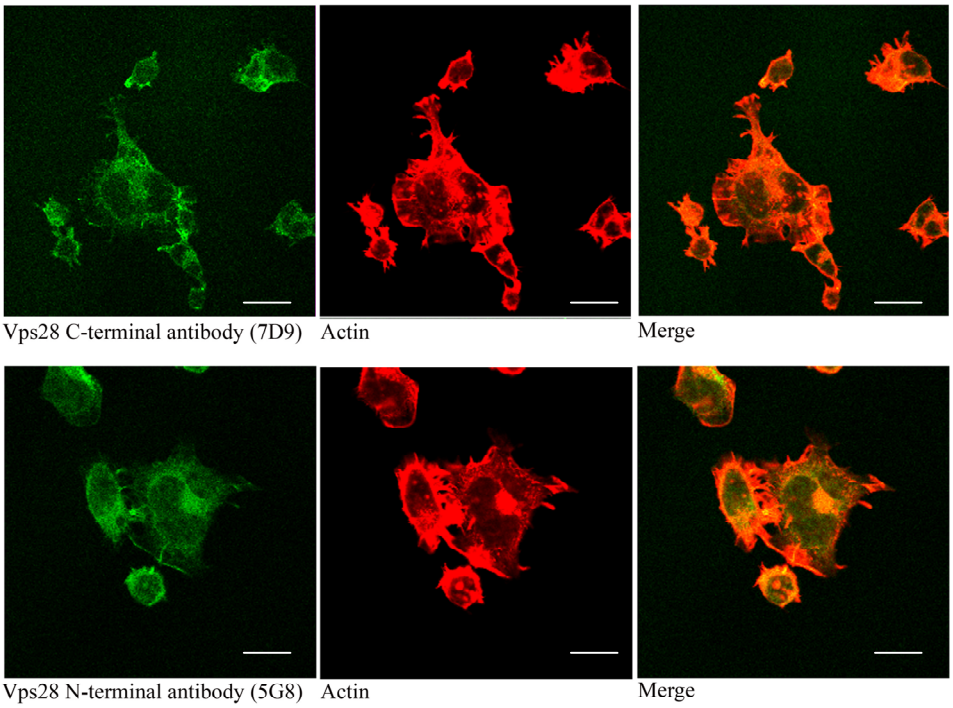


Figure 5.2: Vps28 N- and C-terminal antibody comparison on N1E-115 neuroblastoma cells permanently expressing EphA4 N1E-115 neuroblastoma cells permanently expressing EphA4 receptor were fixed with 4 % PFA and stained with the N- or C-terminal Vps28 antibody and FITC-secondary antibody (green) and F-actin stained with phalloidin-TRITC (red). The scale bar represents 10µm.

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As shown in figure 5.2, both the N- and C-terminal Vps28 antibodies detected endogenous Vps28 in N1E-115 cells permanently expressing EphA4. Both showed some co-localisation with F-actin rich structures. Little difference could be seen between the two antibodies for cell staining.

5.4 Vps28 Interacts with the SH2 Domain of α2-Chimaerin

As previously described, Vps28 was isolated as an interacting partner of α2-chimaerin in a Yeast-2 Hybrid screen. To investigate the interaction in mammalian cells and determine the region of α2-chimaerin interaction with Vps28, COS-7 cells were transfected with HA-tagged Vps28 and either FLAG-tagged α2-chimaerin or truncated α2-chimaerin

fragments. Chimaerin was affinity purified onto FLAG antibody beads and samples were analysed by SDS-PAGE and immunoblotted with HA antibody to identify the binding region.

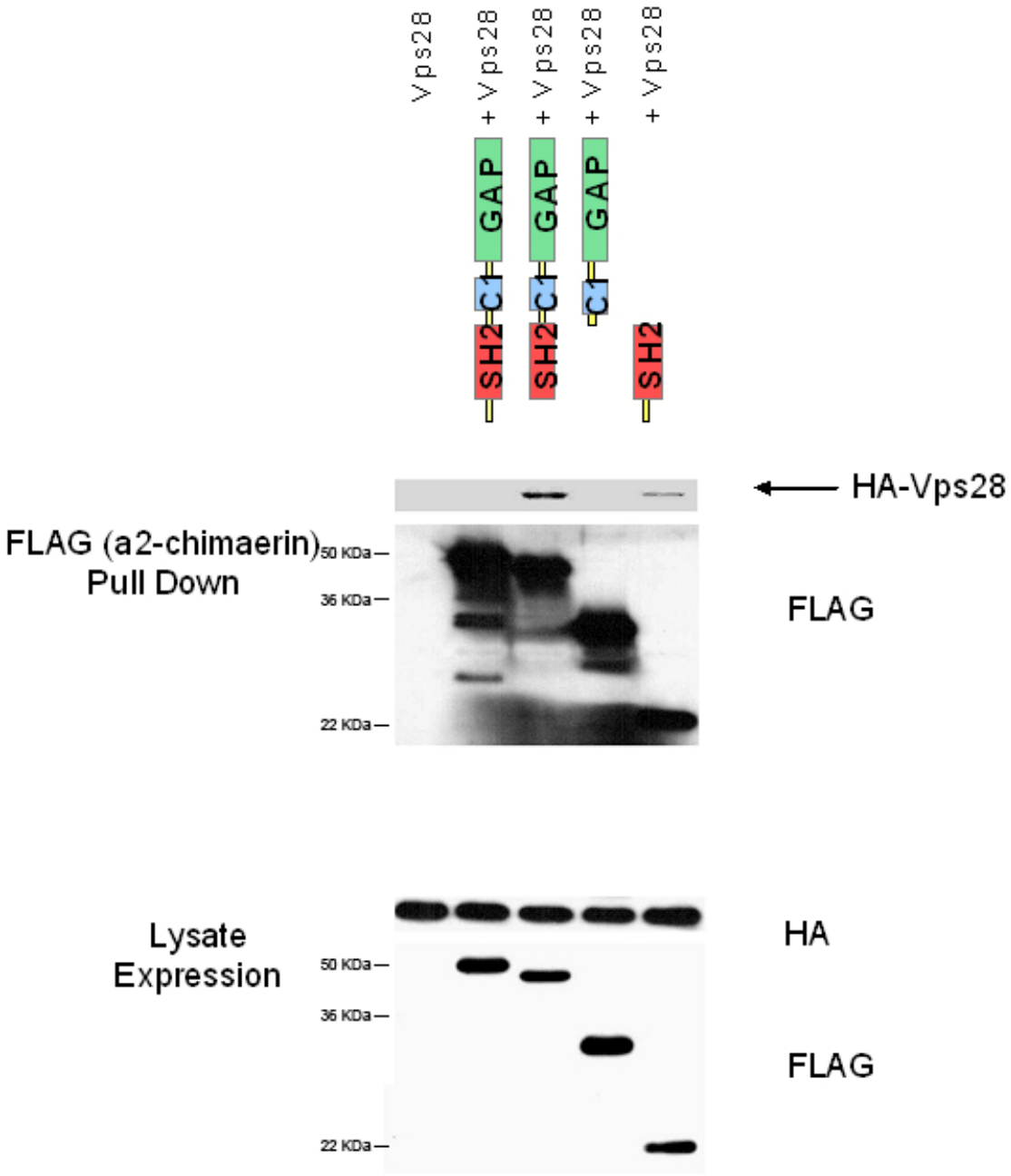

Figure 5.3 Vps28 interacts with α2-chimaerin's SH2 domain COS-7 cells were transfected with FLAG-tagged α2-chimaerin or fragments and HA-tagged Vps28. Cells were lysed and incubated with FLAG antibody beads to pull down

FLAG α2-chimaerin. Samples were analysed by SDS-PAGE and immunodetected with HA and FLAG antibodies. The figure is a representative blot from two experiments.

Vps28 pulls down with the α2-chimaerin SH2 domain, amino acid residues 1-137. Vps28 also pulls down with the 39-459 construct (Figure 5.3). As described previously, the 39-459 N-terminal deletion construct, alters α2-chimaerin into an open conformation and makes it more available for binding than the wild type. Because α2-chimaerin 39-459 binds Vps28 more strongly, there may be other residues contributing to the interaction than in the α2-chimaerin 1-137 SH2 fragment and this interaction was not tested for phosphotyrosine dependence.

To investigate whether Vps28 and α2-chimaerin co-localise within neurones, 3 *div* hippocampal neurones from E18 rat brains were fixed with 4 % paraformaldehyde and stained for endogenous α2-chimaerin and endogenous Vps28, imaged using an LSM510 confocal microscope.

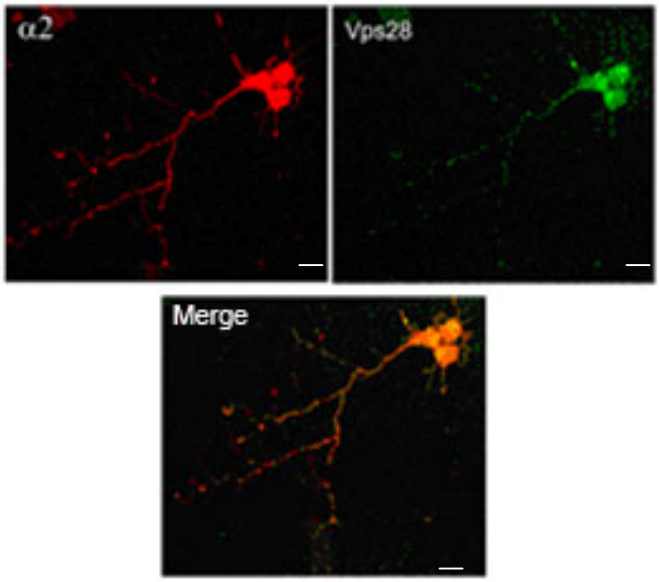

Figure 5.4 Vps28 co-localises with α2-chimaerin in hippocampal neurones 3 *div* hippocampal neurones from E18 rat brains were fixed in 4 % paraformaldehyde and stained with monoclonal Vps28 and rabbit polyclonal α2-chimaerin antibodies for endogenous proteins and secondary antibodies conjugated to FITC (green) and Cy5 (red) respectively and F-actin stained with phalloidin-TRITC. The scale bar represents 10 µm.

Results suggested that endogenous Vps28 and α2-chimaerin co-localise in hippocampal neurones (Figure 5.4).

To investigate Vps28 function in cells, shRNA for Vps28 was produced as described in Chapter 2.0 Methods, using the siSTRIKE U6 Hairpin Cloning System (Promega). To test the Vps28 shRNA, N1E-115 neuroblastoma cells were transfected with the three shRNA sequences for Vps28 or the vector or scrambled sequence controls, after 24 h expression, cells were selected (5 d) with G418 (800 µg/ml). Samples were analysed by SDS-PAGE and immunoblotted with Vps28 antibody and actin antibody as an equalising control.

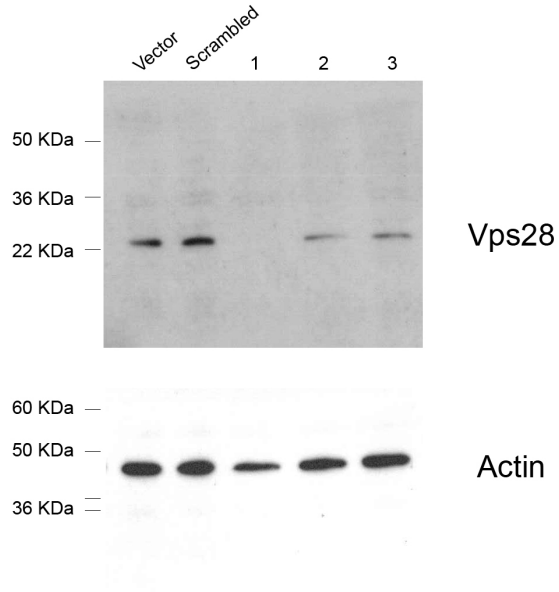

Figure 5.5 Vps28 shRNA sequence 1 knocks down Vps28 N1E-115 neuroblastoma cells were transfected with vector or scrambled shRNA controls or Vps28 shRNA sequences 1, 2 or 3. Cells were treated with 800 µg/ml G418 selection 24 hours later. Cells were lysed after five days and samples were analysed by SDS-PAGE and immunodetected with Vps28 and actin antibodies.

Figure 5.5 shows, all three shRNAs reduced Vps28 levels, with sequence 1 being the most effective.

To investigate whether Vps28 plays a role in endocytosis in the ephrin A1/EphA4 pathway, the Vps28 shRNA sequence 1 was transfected into N1E-115 neuroblastoma cells permanently expressing EphA4.

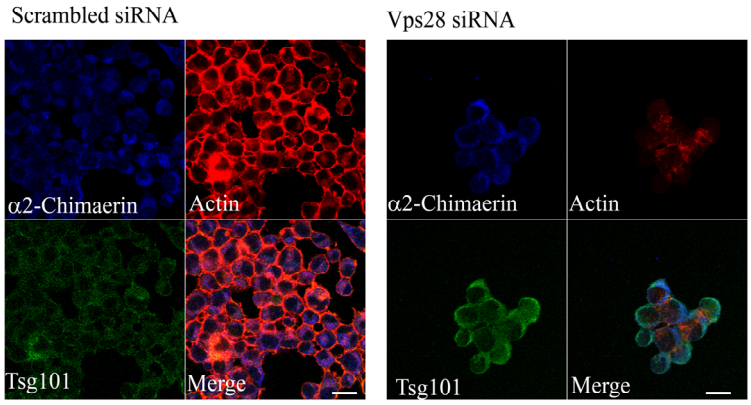

Figure 5.6: Ephrin A1-treated N1E-115 neuroblastoma cells permanently expressing EphA4 transfected with scrambled or Vps28 shRNA N1E-115 neuroblastoma cells permanently expressing EphA4 receptor were transfected with scrambled or Vps28 shRNA sequence 1. Cells were treated for 10 minutes with pre-clustered ephrin A1 prior to being fixed with 4 % PFA and stained with the α2-chimaerin-Cy5 (blue) and ESCRT-I component, Tsg101-FITC (green) antibodies and F-actin stained with phalloidin-TRITC (red). The scale bar represents 10 µm.

Expression of Vps28 shRNA-1 caused extensive cell death and loss of polymerised actin in the few remaining cells, in keeping with an essential role in cell function (Sevrioukov *et. al.*, 2005) (Figure 5.6). Time constraints prevented this from being further investigated.

5.5 Endogenous Associations of α2-Chimaerin in Cortical Neurones

To confirm endogenous associations of α2-chimaerin found in this study, an immunoprecipitation (as described in Chapter 2.0 methods) was carried out using rabbit polyclonal anti-α2-chimaerin antibody that was produced 'in house' (Hall *et. al.*, 2001). Three 10 cm plates of approximately 2 x 10^6 cortical neurones at 7 days *in vitro* were used for each sample. Pre-clustered ephrin A1 treatment was used on one sample to investigate whether ephrin A1/EphA4 forward signalling affected interactions with α2-chimaerin, especially considering the domain shift of EphA4 binding shown in figures

4.1 and 4.2. Neurones were lysed and lysates immunoprecipitated as described in Methods. Samples were subsequently analysed by immunoblotting with antibodies for; (1) α2-chimaerin, to confirm its selective immunoprecipitation; (2) Fyn; (3) EphA4 and (4) Vps28.

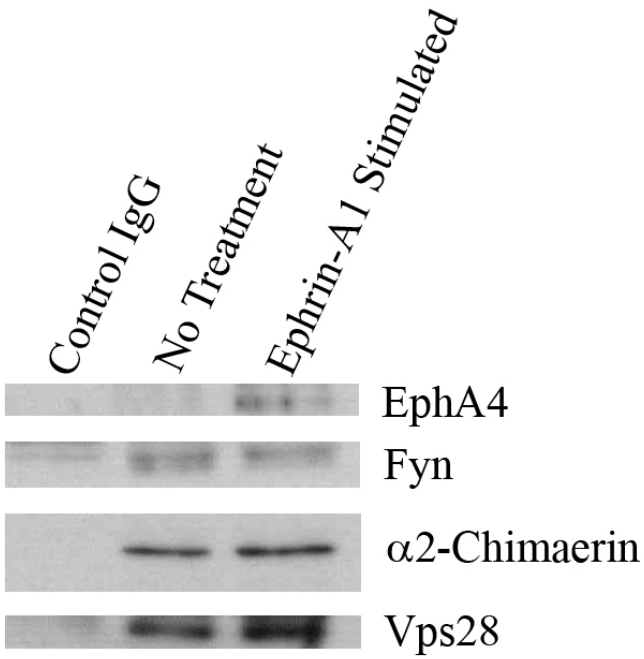

Figure 5.7 α2-Chimaerin immunoprecipitation from cortical neurones 7 div cortical neurones were untreated or treated with 10 minutes of pre-clustered ephrin A1 prior to lysis. Control IgG or rabbit-anti-α2-chimaerin antibody was used to IP α2-chimaerin. Samples were analysed by SDS-PAGE and target antibodies, as indicated, tested to investigate α2-chimaerin endogenous interacting partners.

Results confirmed (Figure 5.7) that α2-chimaerin immunoprecipitated with the rabbit-anti-α2-chimaerin antibody but not the IgG control. Endogenous α2-chimaerin was shown to interact with EphA4, which was only detectable after ephrin A1 treatment. Fyn was also shown to interact endogenously with α2-chimaerin regardless of ephrin A1 treatment. The endogenous association of Vps28 with α2-chimaerin was slightly

increased with ephrin A1 treatment suggesting a possible involvement in receptor stimulated endocytosis. These results demonstrate that EphA4, Fyn and Vps28 are all associated with endogenous α2-chimaerin in cortical neurones. Fyn appears to be a constitutive partner, whilst EphA4 was only detectable as a binding partner after ephrin A1 stimulation. These results are consistent with the over-expression interactions from this study for α2-chimaerin with Fyn and EphA4 (Figures 3.8 and 4.1/2 respectively).

5.6 Identification of α2-Chimaerin Binding Partners in Brain

To identify new α2-chimaerin interacting partners in brain extracts, dual tagged His-Biotin vectors were used. The N-terminal and SH2 domain of α2-chimaerin (1-137) was cloned into the tap-tag vectors as described in Methods. Recombinant α2-chimaerin-SH2 domain His-Biotin was grown in BL-21 cells and the protein purified on a nickel column and subsequently further affinity purified on a streptavidin column (see Methods Chapter 2.0). Synaptosomal membrane extracts (in triton-X100 extraction buffer, see Methods) from 10 d rat brains were used as a source of brain protein. After washing, bound proteins were eluted from the column and analysed by SDS-PAGE as shown in figure 5.8.

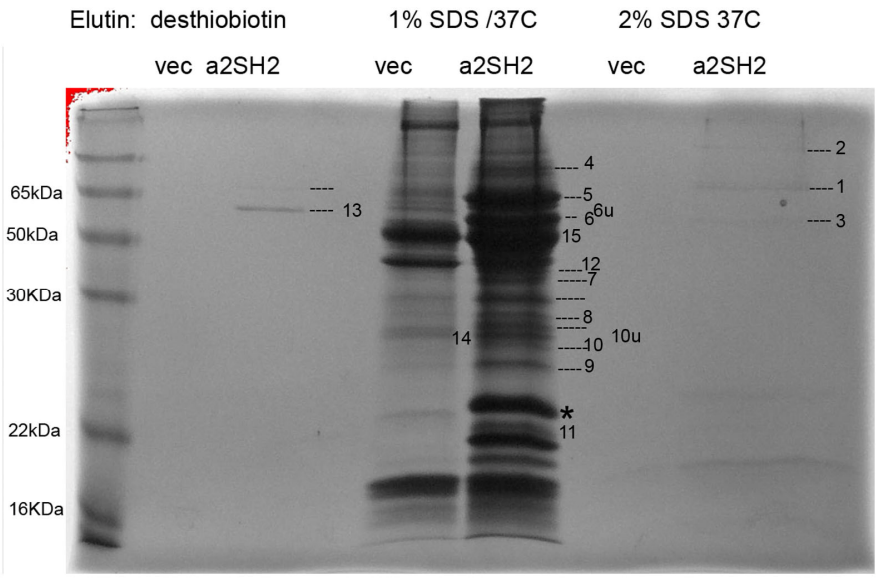

Figure 5.8 α2-Chimaerin pull down from rat synaptosomal brain fraction α2-Chimaerin pHIS-BIOTIN was used as bait, to pull down interacting proteins from rat synaptosomal brain fraction. Samples were analysed by SDS-PAGE and coomassie staining. Bands (numbered) were cut out of the gel to be sent off for mass spectrometry, to investigate α2-chimaerin interacting partners.

After extensive washing and elution with desthiobiotin which did not release bound protein, elution with 1 % SDS at 37 °C was required to elute the protein from the streptavidin column (Figure 5.8). Numbers show bands which were excised from the gel and sent for mass spectrometry analysis.

See appendix for identified proteins, which included;

Band 6 62KD; CRMP 2 (62 kDa);

Band 8 30KD; Prohibitin (2 peptides);

Band 9 26KD; Rabs 2A, 6B, 11B, and 7A;

Tubulin and several mitochondrial proteins were also bound.

5.7 Summary

The results in Chapter 5.0 identify a novel interacting partner of α2-chimaerin, Vps28. Vps28, a member of the ESCRT-I complex, binds active α2-chimaerin, via the SH2 domain (Figure 5.3). Two mouse monoclonal 'in house' antibodies recognising the N-terminal and C-terminal of Vps28 were shown to be suitable for use in cell staining and western blotting. Vps28 shRNA-1 knocks down Vps28 very effectively and caused cell death in N1E-115 neuroblastoma cells permanently expressing EphA4. It may be that Vps28 shRNA sequences 3 and 4 which only partially knocked down Vps28 may be more useful for further studying the function of the Vps28 interaction with α2-chimaerin.

The immunoprecipitation from cortical neurones (Figure 5.7) confirmed Fyn (Shi *et. al.*, 2007), EphA4 (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007) and Vps28 as endogenous α2-chimaerin binding partners. Ephrin A1 treatment enhanced the association with EphA4. The His-Biotin pull down identified the known α2-chimaerin partner CRMP-2 (Brown *et. al.*, 2004) and has also suggested some novel possible α2-chimaerin partners, such as Prohibitin and several Rab proteins. Prohibitin is a mitochondrial protein that has been shown to associate with the plasma membrane, become tyrosine phosphorylated and interact with phosphatidylinositol 3,4,5-triphosphate (Ande and Mishra, 2009). Therefore it is possible that prohibitin could come into contact with α2-chimaerin at the plasma membrane. Rab proteins serve as master regulators in membrane trafficking (Hutagalung and Novik, 2011), thus their interaction with α2-chimaerin implicates some kind of α2-chimaerin involvement in cell trafficking.

Since α2-chimaerin partners include Vps28 and some Rab proteins, it may be involved in endocytosis and vesicle recycling, not previously reported. Unfortunately due to time

restraints the preliminary results in this chapter were unable to be followed up. Interesting future work for chimaerin would include an investigation into the significance of its interaction with Vps28. This is could be the key to downregulation of α2-chimaerin or possible involvement of chimaerin in endocytosis.

Chapter 6.0:

Discussion

6.0 Discussion

In the developing nervous system the formation of neuronal networks requires the accurate navigation of axons and dendrites in response to attractive or repulsive guidance signals. The RhoGTPases regulate changes in the actin cytoskeleton downstream of these signalling molecules, enabling outgrowth and navigation of axons and dendrites (and in cell migration). RhoGAPs (and RhoGEFs), which out-number the GTPases they regulate, are linked with other signal transduction proteins via multiple domains, and function in response to different signals (Bernards, 2003). α-Chimaerins are a major Rac down regulator in neurones and regulators of neuronal morphology, but how they function in pathways linking membrane receptors to the cytoskeleton is only partly understood. At the outset of this study the two isoforms, α1 and α2, had been shown to be involved respectively in pruning of dendrites (Van de Ven *et. al.,* 2005; Buttery *et. al.,* 2006) and in growth cone collapse in sema 3A axonal guidance signalling (Brown *et. al.,* 2004). α2-Chimaerin has subsequently been shown to participate in ephrin signalling, with locomotor defects in mice lacking a functional chimaerin gene (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007; Shi *et. al.*, 2007), and coding mutations in the human gene impeding eye movement (Miyake *et. al.*, 2008).

This study aimed to investigate α2-chimaerin regulation and interactions in neuronal signalling pathways. This led to the identification of α2-chimaerin tyrosine 143 as a Fyn phosphorylation site, and highlighted the importance of Fyn and α2-chimaerin in the sema 3A and ephrin/EphA4 neuronal signalling pathways. As well as Fyn, other novel α2-chimaerin binding partners emerged during and as part of this study, including the

scaffold protein Nck, (Wells *et. al.*, 2006; Chapter 4.0) and Vps28, a component of the ESCRT-I complex.

The RhoGTPases, including Rac, play a vital part in the regulation of the actin cytoskeleton in developing neurones. Rac is essential in guidance signalling, such as in the sema 3A and ephrin/Eph pathways, in which Rac is transiently activated and subsequently downregulated and involved in endocytosis (Jurney *et. al,.* 2002). Rac exchange factor Vav is also involved in endocytosis in ephrin signalling (Cowan *et. al.*, 2005) (and Vav2 regulates EGF receptor endocytosis (Thalappilly *et. al*., 2010)). The regulation of the RacGAP α2-chimaerin in neuronal signalling is tightly controlled, which is essential for regulating neuronal development and outgrowth. The conformation of chimaerins, DAG-binding and involvement of phosphorylation may all play a role in this regulation of chimaerin activity and of its cellular location.

6.1 Biological Function and Regulation of Chimaerin

The crystal structure of β2-chimaerin was determined in 2004 and provided a possible mechanism for chimaerin allosteric activation by lipids (Canagarajah *et. al*., 2004). β2-Chimaerin is held in an autoinhibited, inactive conformation with the C1 domain occluded by the N-terminal, SH2 domain, the linker between the SH2 and C1 domains and the GAP domain (Canagarajah *et. al.*, 2004). The activation of chimaerin by the phorbol ester PMA has been well described (Ahmed *et. al*., 1993). PMA promotes translocation of α2-chimaerin from a predominantly cytosolic location to membranes (Brown et al 2004), although higher concentrations of PMA are required than for the translocation of PKC (Caloca, *et. al.*, 1997). However, DAG although necessary for

chimaerin translocation to membranes, may not be sufficient for its activation (Colón-González *et. al.,* 2008), and in T-cells, translocation of β2-chimaerin is not solely dependant on the C1 domain (Caloca *et. al.,* 2008).

The C1 domain is not accessible to DAG when β2-chimaerin is in an inactive state, and this has also been suggested for other non-kinase phorbol ester receptors such as Munc-13, as well as kinase MRCK (Shen *et. al.,* 2005; Choi *et. al.,* 2008). Their activities are tightly controlled and other factors such as post-translational modifications or protein-protein interactions may contribute to exposure of the C1 domain prior to DAG binding. Possibilities explored in this study and suggested by the work of others for chimaerin activation include tyrosine phosphorylation of α2-chimaerin (Wegmeyer *et. al.,* 2007) and β2-chimaerin (Kai *et. al.,* 2007) and also Nck1 binding to α2-chimaerin (Wegmeyer *et. al.,* 2007; Fawcett *et. al.,* 2007).

In EphA4 signalling, tyrosine phosphorylation of α2-chimaerin may be direct, by the EphA4 receptor (Wegmeyer *et. al.,* 2007), which can phosphorylate a2-chimaerin peptides *in vitro* at Y21, Y143 and Y202 (Warner *et. al.,* 2008), and/or indirect, by the non-RTK SFKs (Shi *et. al.,* 2007). EphA4 stimulation by ephrin A1 resulted in tyrosine phosphorylation of α2-chimaerin and increased activity towards Rac1. SFKs were implicated as a2-chimaerin phosphorylation was reduced with the addition of PP2, a SFK inhibitor (Shi *et. al.,* 2007). Results described in this study suggest that whilst tyrosine phosphorylation of α2-chimaerin occurs in the sema 3A and ephrin/EphA4 pathways, it is unlikely that tyrosine phosphorylation alone activates α2-chimaerin. Moreover, EphA4 mediates signalling through PLCγ activation (Zhou *et. al.,* 2007), thereby producing DAG, which may activate α2-chimaerin in this pathway.

Results described also suggest that α2-chimaerin Y143 and possibly Y303 are Fyn phosphorylation sites *in vivo*. The other tyrosine residues investigated by mutation are unlikely phosphorylation sites for Fyn, but it is conceivable that other kinases may phosphorylate at these sites. In neurones additional sites may be phosphorylated based on the reduced phosphorylation of mutants in ephrin-stimulated neurones Y202, Y303 and Y333 could be phosphosphorylation sites as well as tyrosines in the N-terminal region (Wegmeyer *et. al.,* 2007). Wegmeyer *et. al.,* (2007); and Shi *et. al.,* (2007) also reported multiple likely sites that included Y70, Y143 and Y148 in Hek293 cells expressing EphA4.

Protein sequence homology shows that α2-chimaerin Y143 corresponds to Y153 in β2-chimaerin. Similarly to the findings in this study, β2-chimaerin has been shown to be phosphorylated by the SFK Lck (Kai *et. al.,* 2007; Siliceo and Mérida, 2009) at Y153 and/or Y21. Phosphorylation of Y21 inhibited GAP activity and phosphorylation of Y153 had little effect (Kai *et. al.,* 2007), or showed slight inhibition of levels of active Rac (Siliceo and Mérida, 2009). SFKs phosphorylate β2-chimaerin Y21 in response to cell stimulation with EGF, which appears to negatively regulate β2-chimaerin RacGAP activity (Kai *et. al.,* 2007). This site corresponds to Y11 in α2-chimaerin which was not tested in this study, as it did not score as highly as some other tyrosine sites in the online phosphorylation prediction website used (http://www.cbs.dtu.dk/services/NetPhos/). It would therefore be interesting to investigate whether phosphorylation of α2-chimaerin Y11 would have similar effects to that of β2-chimaerin Y21.

In response to T-cell receptor signalling β2-chimaerin Y153 can be phosphorylated by Lck, regulating β2-chimaerin membrane stabilisation and reportedly, negatively

regulating its GAP activity (Siliceo and Mérida, 2009). Whether Fyn phosphorylation could lead to negative regulation of α2-chimaerin could not be reliably determined. The use of a consistent GAP assay would have helped to investigate this possibility, but physiological chimaerin activation in cells would be required (and its possible inhibition in the case of mutated chimaerin) and this would have been difficult to interpret due to the variable expression levels of transfected chimaerin and chimaerin mutants and so was not included.

Some of the tyrosine mutants produced resulted in α2-chimaerin becoming hyper-phosphorylated on co-expression with Fyn in cells stimulated with sema 3A or ephrin A1. This suggests either that their modification could inhibit association with another protein such as a phosphatase for example or that the amino acid substitutions resulted in disruption of α2-chimaerin structure, allowing increased accessibility to Fyn kinase. This is supported by the results in chapter 4.0 showing that phosphorylation of α2-chimaerin was greatly increased on PMA treatment of cells and without any stimulation, little if any phosphorylation occured. This is also consistent with a study that showed certain mutations of β2-chimaerin caused increased lipid association and GAP activity due to irregular C1 and RacGAP domain exposure (Sosa *et. al.*, 2009).

DAG interaction with the C1 domain is clearly an important control mechanism for α2-chimaerin. The generation of DAG in signalling pathways will have an impact on the membrane location of chimaerin and other C1 domain proteins (Caloca *et. al.*, 1999; Caloca *et. al.*, 2001; Giorgione *et. al.*, 2006). Rac can itself act upstream of some isoforms of PLC, in generating DAG, suggesting a possible feedback loop on Rac inactivation (El-Sibai and Backer, 2007). Phorbol ester treatment promoted stabilisation

of endogenous α1-chimaerin, degradation of which involves the unique N-terminal (Marland *et. al.,* 2011), and DAG may also stabilise α2-chimaerin. This suggests a role for DAG in chimaerin stabilisation as well as the already known potential for chimaerin activation (Ahmed *et. al*., 1993).

6.2 Fyn as a regulator of RhoGAPS

Fyn, a member of the SFKs, can be myristoylated and palmitoylated, anchoring it to the plasma membrane and is involved in trafficking (Resh, 1999; Sandilands *et. al.,* 2007; Sato *et. al.,* 2009). This study demonstrated Fyn binding to α2-chimaerin GAP domain and tyrosine phosphorylation of α2-chimaerin by Fyn. These actions of Fyn may regulate α2-chimaerin in terms of stability, membrane or receptor localisation or GAP activity. Fyn regulates a number of proteins involved in actin dynamics and is involved in oligodendroglial maturation and process outgrowth (Osterhout *et. al.,* 1999; Sperber *et. al*., 2001). Fyn has been shown to modulate actin dynamics by activating Cdk5, which in turn phosphorylates WAVE2, downstream of platelet derived growth factor in migrating oligodendrocyte precursor cells (Miyamoto *et. al.,* 2008).

Fyn is known to regulate the activity of other RhoGTPases. Fyn SH2 domain binds p190RhoGAP, found via a yeast two hybrid screen (Wolf *et. al.,* 2001). Fyn activity regulates phosphorylation of p190RhoGAP, leading to an increase in GAP activity and in RhoGDP. Down-regulation of Rho signalling, which is involved in contractility and neurite retraction, via activation of p190RhoGAP, promotes neurite formation (Liang *et. al.,* 2004; Govek *et. al.,* 2005). This also regulates morphological changes in oligodendrocyte differentiation (Wolf *et. al.*, 2001).

Additionally, Fyn can phophorylate p250RhoGAP enhancing its GAP activity and therefore inactivating Rho (Taniguchi *et. al.,* 2003). Conversely, the GAP activity of TCGAP, a Cdc42 GAP and a regulator of insulin-induced glucose transport in adipocytes, is suppressed by Fyn phosphorylation (Liu *et. al.*, 2006). Fyn activity produces a response aiding process outgrowth due to Cdc42 and Rac1 binding GTP and becoming activated (Krämer-Albers and White, 2011). The precise role that Fyn phosphorylation plays in α2-chimaerin function would be an interesting avenue for further study. α2-Chimaerin direct interaction with Fyn may scaffold other interactions in the EphA4 pathway.

6.3 Nck

The adaptor proteins Nck1 and Nck2, comprising of three SH3 domains and an SH2 domain, interact with Eph receptors, including EphA4 (Holland *et. al.,* 1997; Bisson *et. al.,* 2007). Nck1 and 2 also link phosphotyrosine signalling to actin cytoskeleton regulating proteins, (Bladt *et. al.,* 2003) such as N-WASP and the WAVE1 complex (Rivero-Lezcano *et. al.,* 1995; Eden *et. al.,* 2002). Nck1 has been shown to bind α2-chimaerin (Wells *et. al.,* 2006). In addition, α2-chimaerin, but not α1-chimaerin was demonstrated to bind both Nck1 and Nck2, requiring α2-chimaerin SH2 domain (N-terminal 1-183 amino acids) and one of the three SH3 domains of Nck (Wegmeyer *et. al.,* 2007). This study established that the first 1-39 amino acid residues of α2-chimaerin contain a Nck1 binding site. The SH3 domain of Nck1 that binds α2-chimaerin was not mapped during this study, but was determined at the same time by collaborators (Sit Soon Tuck and Ed Manser, Singapore, personal communication).

Results also suggested that Nck may function as a complex with α2-chimaerin and Fyn since all three proteins, α2-chimaerin, Fyn and Nck, were able to associate (Chapter 4.0). In addition, when Nck was present, α2-chimaerin could bind Fyn effectively in the absence of PMA. This is in concurrence with Wegmeyer *et. al.,* (2007) who suggested that α2-chimaerin and Nck may act together to balance Rac activity. Within the limitations of the RacGTP assay and variable expression levels of the different proteins, these results indicated that Nck may either limit chimaerin RacGAP activity or act downstream of EphA4 in promoting Rac activity, counterbalanced by chimaerin (Wegmeyer *et. al.,* 2007). Nck2 is also involved in ephrin B3 reverse signalling, regulating interactions with Dock180 and PAK, altering actin reorganization and axon pruning (Xu and Henkemeyer, 2009). Nck adaptors are implicated in linking phosphotyrosine signals to cytoskeleton reorganization and are required for the development of normal limb movement, since Nck1 (-/-) mice with Nck2 knockout in the nervous system showed a similar phenotype to EphA4 (-/-) mice (Fawcett et al, 2007; Kullander *et. al.*, 2003). Nck may also be involved in EphA4-induced loss of adhesion required in cell sorting in development (Bisson et. al., 2007), and interact with EphA3 phosphotyrosine residues in cell migration and process retraction (Hu *et. al.*, 2009). The mounting supporting evidence suggests that Nck adaptors are vital in neuronal signalling pathways and development.

It has been reported that in T-cell receptor activity, Nck binding to the CD3 subunits of T-cell receptors inhibits phosphorylation by Lck or Fyn, thereby acting as a down regulator of receptor signalling (Takeuchi *et. al*., 2009). Currently there is no evidence for Nck inhibiting EphA4 or α2-chimaerin phosphorylation by Fyn, however, it is

possible that similar regulatory mechanisms could be involved in ephrin/EphA4 signalling. Results in this study demonstrated that Nck could be phosphorylated by Fyn, which could alter its subsequent interactions.

Further studies would be of interest to determine whether Nck1, Nck2 or both are involved in ephrin A1/EphA4 signalling and whether regulation by tyrosine phosphorylation is involved. This could be done using selective Nck1 and 2 knock-down in neurones or in the N1E-115 neuroblastoma cell line permanently expressing EphA4 and testing the response in an ephrin A1 induced collapse assay. Single point mutations of predicted phosphorylation sites would aid investigation into its regulation by tyrosine phosphorylation. Nck1 and 2 are adaptor proteins that interact with integrins and GIT1 (Zhao *et. al.,* 2000; Buday *et. al.,* 2002), and are involved in the activation of N-WASP and related proteins, promoting the polymerization of F-actin (Tomasevic *et. al.,* 2007). Nck SH3 domains are important in these functions and interaction with chimaerin may conceivably down-regulate this function of Nck.

6.4 Role of α2-chimaerin Domains

The role of α2-chimaerin SH2 domain is as yet undefined. Several binding partners of α2-chimaerin have been identified that interact with the SH2 domain. These include CRMP-2, as well as Vps28 which was identified as a binding partner during the course of this study. EphA4 under certain conditions (such as ephrin stimulation (Figure 4.2; Beg *et. al.,* 2007; Wegmeyer *et. al.,* 2007)) may also bind α2-chimaerin SH2 domain. The results in chapter 4.0 are consistent with the other reports suggesting that there are 2 binding sites for EphA4 in chimaerin (Beg *et. al.,* 2007; Wegmeyer *et. al.,* 2007). Nck1

was thought to bind α2-chimaerin SH2 region (and was unaffected by a mutation abolishing phosphotyrosine binding), however, pull down experiments with α2-chimaerin fragments in this study have shown that it more likely binds the N-terminal region adjacent to the SH2 domain, a region not present in α1-chimaerin. This further demonstrates the importance of the unique N-terminal as well as other domains in control mechanisms for chimaerin, as with other tightly regulated small GTPase GAPs. It has been suggested that the N-terminal GTP binding domain of p190RhoGAP plays an auto regulatory role (Tatsis *et. al.,* 1998).

As previously described, the C1 domain is very important in altering conformation and in membrane translocation of α2-chimaerin, affecting the accessibility of α2-chimaerin binding and phosphorylation sites. It has recently been reported that phosphorylation by PKC_{δ} of serine 169 in the linker region between the SH2 and C1 domains of β2-chimaerin, prevents membrane translocation, thus keeping β2-chimaerin in the cytosol and away from membrane-associated Rac (Griner *et. al.,* 2010). Since PKC is also regulated by DAG, it would be interesting to investigate whether PKC or other serine/threonine phosphorylation in α2-chimaerin had the same inhibiting effect. However, this site is not conserved in the α2-chimaerin linker region suggesting that the two proteins may be differently regulated.

The GAP domain of chimaerin regulates Rac1 function, which affects morphology. Binding of α1-chimaerin to the NMDA receptor NR2A subunit in a phorbol ester-dependent manner, has been shown in neuronal dendrites to inhibit new spine formation and prune existing spines. This process was shown to require a functional α1-chimaerin GAP domain and is therefore mediated by Rac1 activity, which regulates F-actin in these

structures (Van de Ven *et. al.,* 2005; Buttery *et. al.,* 2006). Additionally, α1-chimaerin protein levels were reported to be controlled by synaptic activity and an increase in expression resulted in pruning of dendritic spines and branches in cultured Purkinje and hippocampal neurones, requiring both DAG binding and RacGAP activity. Results in chapter 3.0 show over-expression of α2-chimaerin mutated to inactivate the GAP domain (R304G) in rat hippocampal neurones (Chapter 3.0), which resulted in altered morphology, with some spread growth cones. Over-expression of wild type α2-chimaerin promoted increased branching and axon number, whilst α2-chimaerin SH2 domain inactivating mutant (R73L) appeared to be without any effect, and surprisingly some phosphotyrosine mutants strongly promoted branching. This may indicate that α2-chimaerin is regulated by multiple interactions and modifications involving all three domains and with subtle effects on development of neuronal structure.

6.5 Other Functions

In addition to the roles of chimaerin already mentioned, during the course of this study other emerging functions have become apparent. α2-Chimaerin has been demonstrated to be important in ocular motor neurone axon path finding, where gain-of-function missense mutations in CHN1 cause a variant form of the congenital eye movement disorder Duane's retraction syndrome (Miyake *et. al.,* 2008). Very recently α-chimaerin has been identified as having a possible role in the postnatal period of visual cortical plasticity in cats (Yang *et. al.,* 2011). This evidence supports the notion that conformational changes of chimaerins, such as possibly occurs with the hyperphosphorylated tyrosine mutants, can lead to functional changes or disruption.

Results from this study suggest a possible new role for α2-chimaerin in endocytosis. Ectopic expression of the GAP domain of α2-chimaerin was previously shown to inhibit cytoskeletal response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine and colony stimulating factor-1, thereby preventing phagocytosis, in which Rac is a key regulator (Cox *et. al.,* 1997). Interestingly, Fyn is present in RhoD (an endosomal marker) positive endosomes (Sandilands *et. al.,* 2007). The mass spectrometry data from this study indicates that some Rabs, small G-proteins highly involved in regulating vesicular transport, are also likely to be associated with α2-chimaerin. These potential α2-chimaerin partners function in neurones (Ng and Tang, 2008); Rabs 2A and 6A are involved in golgi transport (Ayala *et. al.,* 1990; Shetty *et. al.,* 1998), and Rabs 7A and 11A are involved in endosomal sorting (Saxena *et. al.,* 2005; Satoh *et. al.,* 2005; Shirane and Nakayama, 2006).

Results from this study also suggest a role for α2-chimaerin in the ubiquitin proteasome system, with the SH2 domain associating with Vps28, a component of the ESCRT-I complex. This is a novel finding not yet supported by other studies, Vps28 was isolated as a yeast two hybrid partner of α2-chimaerin (C. Monfries, C. Hall, personal communication) and endogenous Vps28 could be immunoprecipitated with chimaerin (Chapter 5.0). Vps28 interacts with Tsg101 and in cells the interaction stabilises its expression levels (Bishop and Woodman, 2001). Vps28 C-terminal connects with ESCRT-II (Kostelansky *et. al.,* 2006)

In Drosophila Vps28 knockdown has been shown to alter the actin cytoskeleton (Sevrioukov *et. al.,* 2005) and interestingly another RhoGAP, BCR interacts with Tsg101 (Olabisi *et. al.,* 2006). Thus RhoGAPs may link regulation of the actin cytoskeleton

through Rac and Rabs to vesicle formation and trafficking, as reported for P50RhoGAP (Sirokmany *et. al.*, 2006). Additionally, it has been suggested that Eps8 protein also connects receptor signalling through Rac to cell trafficking through Rab5 (Lanzetti *et. al.*, 2000), suggesting a common link between the two processes, possibly involving Rac-dependent actin rearrangements and/or receptor recycling. In addition, Rab35 has been reported to regulate cell shape and neurite outgrowth, indicating a wider role for Rabs other than in cell trafficking (Chevallier *et. al.*, 2009).

6.6 Models for α2-Chimaerin in Neuronal Collapse Pathways

The results from this study, taken together with published research have enabled models to be developed of how α2-chimaerin may function in growth cone collapse in axonal guidance signalling pathways. The SH2 and GAP domains of α2-chimaerin were shown to be essential in the sema 3A growth cone collapse pathway (Brown *et. al.*, 2004). The model in figure 6.1 illustrates how α2-chimaerin may act in the pathway stimulated by sema 3A through interaction with Cdk5 and its substrate CRMP2 (Brown *et. al.*, 2004). In this study, α2-chimaerin was shown to be phosphorylated by Fyn, also a component of this pathway (Sasaki *et. al.*, 2002). Phosphorylation of Cdk5 primes for phosphorylation of CRMP2 by GSK3β, a central kinase in the sema 3A pathway (Eickholt *et. al.*, 2002), regulating activity of microtubule associated proteins (Gu and Ihara, 2000). This transmits the signal to affect microtubule dynamics, whilst regulation of Rho GTPases and associated proteins also alter the actin cytoskeleton. It is possible that elements from this model may also function downstream of EphA4, but this is yet to be demonstrated. Fyn participates in sema 3A guidance and activates Cdk5 by phosphorylating N-terminal

Y15 (Sasaki *et. al.*, 2002; Morita *et. al.*, 2006) and most likely phosphorylates α2-chimaerin in this pathway. However, since phosphorylation is greatly enhanced on PMA treatment, which would allow access to buried tyrosine residues, Fyn phosphorylation alone is unlikely to activate α2-chimaerin in the pathway and DAG probably has a key role in activating α2-chimaerin prior to phosphorylation.

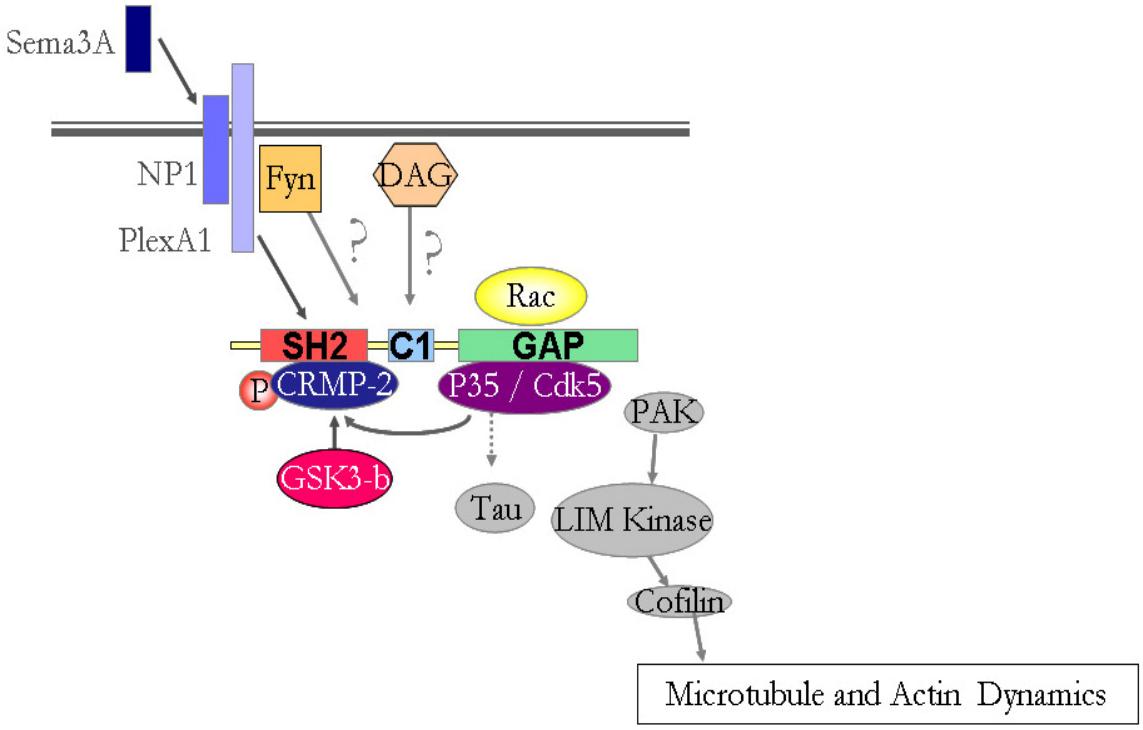

Figure 6.1: Model for α2-chimaerin in the sema 3A pathway Sema 3A signals across the plasma membrane though the NP1/PlexA1 receptor. Fyn and DAG may play a role in the pathway; Fyn activates Cdk5. Rac1 and p35/Cdk5 bind the GAP domain of α2-chimaerin. p35/Cdk5 phosphorylates CRMP-2 causing phosphorylation by GSK3-β. CRMP-2 binds α2-chimaerin SH2 domain. The signal is then cascaded down to affect microtubule and actin dynamics, possibly involving Tau, PAK, LIM Kinase and Cofilin.

α2-Chimaerin was more recently shown to be involved in EphA4 signalling (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007; Shi *et. al.*, 2007). The chimaerin

interactions previously identified in the sema 3A pathway may also be involved in EphA4 signalling. However, results from this study combined with the work of others have indicated that other partners may play a key role, including Nck and Fyn (Wegmeyer *et. al.*, 2007; Shi *et. al.*, 2007). Possible mechanisms are shown in figure 6.2, of how α2-chimaerin may function in growth cone collapse pathways in neurones downstream of EphA4.

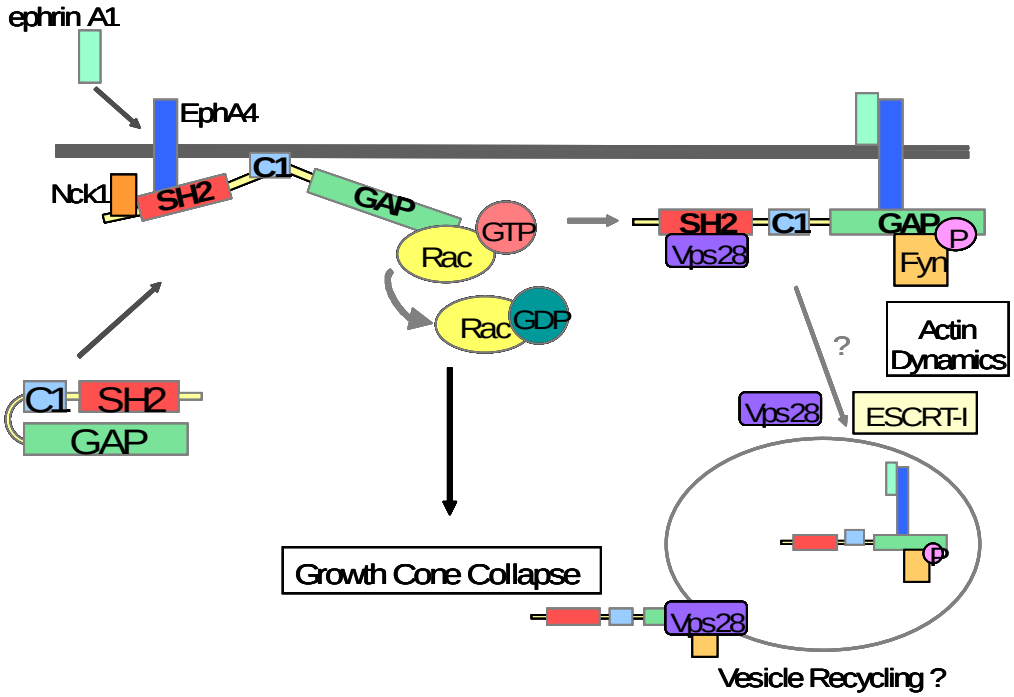

Figure 6.2: Model for α2-chimaerin in the ephrin A1/EphA4 pathway This model for α2-chimaerin proposes that following stimulation of EphA4 by the ligand ephrin A1, α2-chimaerin translocates from the cytosol to membranes, stimulated either by DAG and/or interaction with proteins such as Nck, EphA4 receptor, Fyn, or another mechanism. α2-Chimaerin C1 domain interacts with the membrane and SH2 domain may directly bind EphA4. Activated α2-chimaerin down-regulates Rac1-GTP initiating a collapse response. Fyn binds the GAP domain and phosphorylates α2-chimaerin SH2-C1 linker altering further interactions (or marking it for degradation). EphA4 may also bind to α2-chimaerin GAP domain to inhibit further signalling, leaving the SH2 domain open for new interactions. On termination of signalling, the receptor complex may enter the endocytosis pathway and pass into the ESCRT sorting pathway. ESCRT 1 component Vps28 binds the SH2 domain of α2-chimaerin linking it with associated actin remodelling. The receptor complex may be degraded or recycled back to the plasma membrane.

α2-Chimaerin may translocate from the cytosol to the membrane, stimulated either by DAG and/or another interaction. For example, Nck1 binding to α2-chimaerin N-terminal in response to receptor stimulation at the plasma membrane, involving tyrosine phosphorylation and SH2 interaction. Whether the SH2 domains of Nck, Fyn or α2-chimaerin interact directly with EphA4 is not established. The C1 domain interacts with the membrane, and α2-chimaerin SH2 domain may directly bind EphA4. α2-Chimaerin becomes activated and down regulates Rac1 to initiate a collapse response. Subsequently/concurrently Fyn may bind and phosphorylate α2-chimaerin Y143 in C1-SH2 linker (and possibly the GAP domain at Y303) altering its interactions, activity or to mark it for degradation. EphA4 may bind to α2-chimaerin GAP domain to inhibit signalling, leaving the SH2 domain open for binding. The whole receptor complex including α2-chimaerin could then enter the endocytosis pathway where it may be degraded or recycled back to the plasma membrane.

The ESCRT-I component Vps28 can bind α2-chimaerin SH2 domain. Whilst chimaerin may also be a cargo protein, it is more likely that it mediates regulation of Rac and co-ordination with Rab proteins (which are also chimaerin binding targets (Chapter 5.0)) affecting vesicle formation at the plasma membrane and trafficking as early endosomes. Although this remains to be investigated, the results described here and several key papers published during this study have contributed to a greater understanding of α2-chimaerin in axonal guidance signalling and its involvement in ephrin/EphA4 neuronal growth cone collapse (Wegmeyer *et. al.*, 2007; Beg *et. al.*, 2007; Iwasato *et. al.*, 2007; Shi *et. al.*, 2007; Fawcett, *et. al.*, 2007).

6.7 Future work

There are many possible areas of investigation which could be expanded upon. The tyrosine mutants constructed in this study and available deletion mutants of α2-chimaerin were very useful for mapping sites of α2-chimaerin modification and its interactions with the binding partners identified during this study. However, time constraints prevented cloning of deletion or domain inactivating mutants of all the α2-chimaerin partners, to map the regions of binding, especially of Fyn, Nck and EphA4. Equivocal results were obtained for the latter (Beg *et. al*., 2007; Wegmeyer *et. al*., 2007) It is not established whether the kinase domain, SH2 or SH3 domain of Fyn binds chimaerin GAP domain. The role of Fyn phosphorylation of α2-chimaerin is also an interesting area which requires further investigation. A workable assay for assessing GAP activity may help to establish whether and how Fyn phosphorylation regulates α2-chimaerin activity. This would require a sensitive and rapid assay, and the conventional RacGTP binding assays have not proved to be as sensitive for down-regulation by GAP activity as for GEFs.

It was unfortunate that the Vps28 section of this study came so near to the end, as investigating the function of this novel α2-chimaerin binding partner would be of real interest. A role in endocytosis has not been reported for α2-chimaerin. In view of the links between Rac and Rab small GTPases in endocytosis (Bhattacharya *et. al.*, 2004), this interaction should be pursued. Ideally a cell assay would need to be established with fluorescent tags which could enable visualisation of α2-chimaerin localisation in endocytosis. The effects of chimaerin knockdown and chimaerin mutants on the formation of MVB and cargo trafficking could also be investigated. Although the Vps28 shRNA which most effectively eliminated Vps28 led to cell apoptosis, there were other

shRNA sequences which only caused a partial knockdown of Vps28, which may be of some use for future investigations. Interestingly, Tsg101 knockdown is also lethal and these two proteins interact directly (Ruland *et. al*., 2001; Bishop and Woodman, 2001). Further investigation of the interaction of chimaerin with the rab proteins identified by the proteomic approach in chapter 5.0 and their role in endocytosis and vesicle trafficking would be interesting in relation to this.

A key issue highlighted by the number of binding partners investigated, is under what circumstances chimaerin interacts with these potential multiple endogenous partners. Chimaerin is expressed as 2 isoforms and some partners may interact with only α2-chimaerin via the N-terminal and SH2 domains, but α2-chimaerin is expressed in adult neurones as well as throughout development, and in testis (Hall *et. al*., 1993). Further studies involving primary neurones, including dendritic morphology and mass spectrometry of interacting partners, would help to investigate phosphorylation, endogenous interactions and functions of α2-chimaerin within specific neuronal signalling pathways and during development.

The signalling transducers that link membrane receptors and the RhoGTPases and co-ordinate their activities are still under much investigation. For example, the GEF ephexin1 has been shown to be an essential mediator for EphA-dependent growth cone collapse through the activation of RhoA and the inhibition of Rac1 (Shamah *et. al.*, 2001; Sahin *et. al.*, 2005). The regulation of ephexin1 by EphA4 is dependent, among others, upon SFKs and Cdk5, both of which are also involved in signalling with α2-chimaerin (Qi *et. al.*, 2004), further supporting their roles together in the EphA4 pathway.

6.8 Conclusions

This study has identified α2-chimarin Y143 as a Fyn phosphorylation site and that Fyn interacts with α2-chimaerin GAP domain. These findings have added to the evidence that Fyn has a key role in signalling pathways, including the sema 3A and ephrin A1/EphA4 pathways looked at in this study. This investigation has also demonstrated that Nck binds to α2-chimaerin N-terminal and that a complex may be formed with α2-chimaerin acting as a scaffolding protein for Nck and Fyn. EphA4 may interact with α2-chimaerin at two different sites dependent upon its activation status, which could explain the differences found in other studies as mentioned above (Chapter 4.0). Fyn may generate phosphotyrosine binding sites for other interactions in EphA4 signalling and since Fyn, Nck and α2-chimaerin all have SH2 domains and Nck also has three SH3 domains (and Fyn has an SH3), multiple interactions may participate in the receptor complex. Novel binding partners of α2-chimaerin identified in this study, Vps28 and Rab proteins, suggest a possible role or method of degradation/recycling for α2-chimaerin in endocytosis.

This study investigated α2-chimaerin as a piece of this complex puzzle and has tried to demonstrate where existing and novel α2-chimaerin partners may fit into neuronal collapse pathways. Chimaerins are fascinating proteins with many emerging functions. This study contributes in a small way to aiding the understanding of the complex signalling pathways involved in neuronal development.

Chapter 7.0:

References

7.0 References

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Appendix- Mass Spectrometry Data

Crmp-2

gi|40254595 (100%), 62,277.9 Da
dihydropyrimidinase-like 2 [Mus musculus], gi|157786744|ref|NP_001099187.1| dihydropyrimidinase-like 2 [Rattus
2 unique peptides, 2 unique spectra, 2 total spectra, 23/572 amino acids (4% coverage)

M S Y Q G K K N I P R I T S D R L L I K G G K I V N D D Q S F Y A D I Y M E D G
L I K **Q I G E N L I** **V P G G V K** T I E A H S R M V I P G G I D V H T R F Q M P D
Q G M T S A D D F F Q G T K A A L A G G T T M I I D H V V P E P G T S L L A A F
D Q W R E W A D S K S C C D Y S L H V D I T E W H K G I Q E E M E A L V K D H G
V N S F L V Y M A F K D R F Q L T D S Q I Y E V L S V I R D I G A I A Q V H A E
N G D I I A E E Q Q R I L D L G I T G P E G H V L S R P E E V E A E A V N R S I
T I A N Q T N C P L Y V T K V M S K **S A** **A E V I A Q A R** K K G T V V Y G E P I T
A S L G T D G S H Y W S K N W A K A A A F V T S P P L S P D P T T P D F L N S L
L S C G D L Q V T G S A H C T F N T A Q K A V G K D N F T L I P E G T N G T E E
R M S V I W D K A V V T G K M D E N Q F V A V T S T N A A K V F N L Y P R K G R
I S V G S D A D L V I W D P D S V K T I S A K T H N S A L E Y N I F E G M E C R
G S P L V V I S Q G K I V L E D G T L H V T E G S G R Y I P R K P F P D F V Y K
R I K A R S R L A E L R G V P R G L Y D G P V C E V S V T P K T V T P A S S A K
T S P A K Q Q A P P V R N L H Q S G F S L S G A Q I D D N I P R R T T Q R I V A
P P G G R A N I T S L G

Prohibitin

gi|62664759 (100%), 27,717.2 Da
PREDICTED: similar to prohibitin [Rattus norvegicus], gi|109508393|ref|XP_001060250.1| PREDICTED: similar to prol
2 unique peptides, 2 unique spectra, 2 total spectra, 20/253 amino acids (8% coverage)

M A A K V F E S I G K F G L A L A V A G G V V N S A L Y N V D A A T D R F R G V
Q D I V V G E G T H F L I P W V Q K P I I F D C R S R P R K V P V I T G S K **D L**
Q N V N I T L R I L F R P V A S Q L P R I Y T S I G E D Y D E R V L P S I T T E
I L K S V V A R **F D** **A G E L I T Q R** E L V S R Q V S D D L T E R A A T F G L I L
D D V S L T H L T F G K E F T E A V E A K Q V A Q Q E A E R A R F V V E K A E Q
Q K K A A I I S A E G D S K A A E L I A N S L A T A G D G L I E L R K L E A A E
D I A Y Q L S R S R N I T

Rabs

gi|157817539 (100%), 19,878.0 Da
RAB6B, member RAS oncogene family [Rattus norvegicus], gi|194221614|ref|XP_001496020.2| PREDICTED: similar to
4 unique peptides, 4 unique spectra, 4 total spectra, 41/175 amino acids (23% coverage)

M Y D S F D N T Y Q A T I G I D F L S K T M Y L E D R T V R **L Q L W D T A G Q E**
R F R **S L I P S Y I** **R** D S T V A V V V Y D I T N L N S F Q Q T S K W I D D V R T
E R **G S D V I I M** L **V G N K** T D L A D K R **Q I T I E E G E Q** **R** A K E L S V M F I
E T S A K T G Y N V K Q L F R R V A S A L P G M E N V Q E K S K E G M I D I K L
D K P Q E P P A S E G G C S C

gi|14249144 (100%), 24,489.0 Da
RAB11B, member RAS oncogene family [Rattus norvegicus], gi|78369332|ref|NP_001030468.1| RAB11B, member RAS
2 unique peptides, 2 unique spectra, 2 total spectra, 19/218 amino acids (9% coverage)

M G T R D D E Y D Y L F K V V L I G D S G V G K S N L L S R F T R **N E F N L E S**
K S T I G V E F A T R S I Q V D G K T I K **A Q I W D T A G Q** **E R** Y R A I T S A Y
Y R G A V G A L L V Y D I A K H L T Y E N V E R W L K E L R D H A D S N I V I M
L V G N K S D L R H L R A V P T D E A R A F A E K N N L S F I E T S A L D S T N
V E E A F K N I L T E I Y R I V S Q K Q I A D R A A H D E S P G N N V V D I S V
P P T T D G Q K P N K L Q C C Q N L

gi|13027392 (100%), 23,504.0 Da
RAB7A, member RAS oncogene family [Rattus norvegicus], gi|1710001|sp|P09527.2|RAB7A_RAT RecName: Full=Ras-r
2 unique peptides, 2 unique spectra, 2 total spectra, 23/207 amino acids (11% coverage)

M T S R K K V L L K V I I L G D S G V G K T S L M N Q Y V N K K F S N Q Y K A T
I G A D F L T K E V M V D D R L V T M Q I W D T A G Q E R **F** **Q S L G V A F Y R** G
A D C C V L V F D V T A P N T F K T L D S W R D E F L I Q A S P R **D P E N F P F**
V V L G N K I D L E N R Q V A T K R A Q A W C Y S K N N I P Y F E T S A K E A I
N V E Q A F Q T I A R N A L K Q E T E V E L Y N E F P E P I K L D K N E R A K A
S A E S C S C

Tubulin

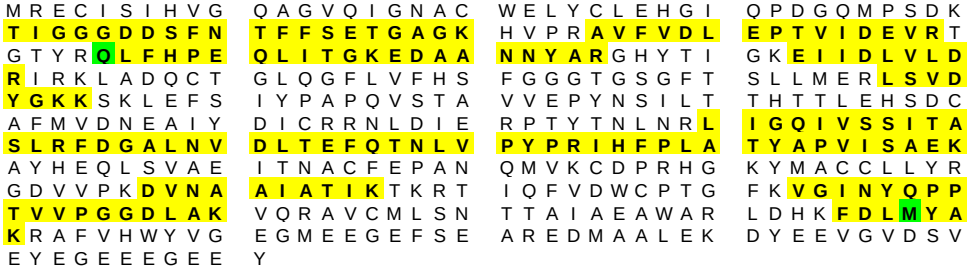
gi|21746161 (100%), 49,953.1 Da
tubulin, beta [Mus musculus], gi|29788768|ref|NP_821080.1| tubulin, beta 2B [Homo sapiens], gi|1103476
10 unique peptides, 10 unique spectra, 12 total spectra, 106/445 amino acids (24% coverage)

M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I D P T G S Y H G D S
D L Q L E R **I N V Y** **Y N E A T G N K** Y V P R **A I L V D L E P** **G T M D S V R** S G P
F G Q I F R P D N F V F G Q S G A G N N W A K G H Y T E G A E L V D S V L D V V
R K E S E S C D C L Q G F Q L T H S L G G G T G S G M G T L L I S K I R E E Y P
D R **I M N T F S V M** **P S P K** V S D T V V E P Y N A T L S V H Q L V E N T D E T Y
C I D N E A L Y D I C F R T L K L T T P T Y G D L N H L V S A T M S G V T T C L
R **F P G Q L N A D L** **R K L A V N M V P F** **P R** L H F F M P G F A P L T S R G S Q Q
Y R **A L T V P E L T** **Q Q M F D S K** N M M A A C D P R H G R **Y** **L T V A A I F R** G R
M S M K **E V D E Q M** **L N V Q N K** N S S Y F V E W I P N N V K T A V C D I P P R G
L K M S A T F I G N S T A I Q E L F K R **I S E Q F T A M F R** R K A F L H W Y T G
E G M D E M E F T E A E S N M N D L V S E Y Q Q Y Q D A T A D E Q G E F E E E E
G E D E A

gi|12963615 (100%), 50,418.7 Da
tubulin, beta 3 [Mus musculus], gi|145966774|ref|NP_640347.2| tubulin, beta 3 [Rattus norvegicus], gi|204
5 unique peptides, 5 unique spectra, 5 total spectra, 127/450 amino acids (28% coverage)

M R E I V H I Q A G Q C G N Q I G A K F W E V I S D E H G I D P S G N Y V G D S
D L Q L E R **I S V Y** **Y N E A S S H K** Y V P R **A I L V D L E P** **G T M D S V R** S G A
F G H L F R P D N F I F G Q S G A G N N W A K G H Y T E G A E L V D S V L D V V
R K E C E N C D C L Q G F Q L T H S L G G G T G S G M G T L L I S K V R E E Y P
D R **I M N T F S V V** **P S P K** V S D T V V E P Y N A T L S I H Q L V E N T D E T Y
C I D N E A L Y D I C F R T L K **L A T P** **T Y G D L N H L V S** **A T M S G V T T S L**
R F P G Q L N A D L **R K L A V N M V P F** **P R** L H F F M P G F A P L T A R G S Q Q
Y R **A L T V P E L T** **Q Q M F D A K** N M M A A C D P R H G R Y L T V A T V F R G R
M S M K E V D E Q M L A I Q S K N S S Y F V E W I P N N V K V A V C D I P P R G
L K **M S S T F I G N** **S T A I Q E L F K** R **I S E Q F T A M F R** R K A F L H W Y T G
E G M D E M E F T E A E S N M N D L V S E Y Q Q Y Q D A T A E E E G E M Y E D D
D E E S E A Q G P K

gi|223556 (100%), 50,241.8 Da
tubulin alpha
12 unique peptides, 13 unique spectra, 14 total spectra, 159/451 amino acids (35% coverage)



Mitochondrial

gi|13324704 (100%), 24,297.4 Da
GrpE-like 1, mitochondrial [Rattus norvegicus], gi|6226823|sp|P97576.2|GRPE1_RAT RecName: Full=GrpE protein ho
12 unique peptides, 13 unique spectra, 15 total spectra, 120/217 amino acids (55% coverage)